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A NEW METHOD OF CULTURING SPUTUM ON SOLID MEDIA USING CARBON DIOXIDE FOR THE ISOLATION OF PNEUMOCOCCI.

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UNTIL the present time the ordinary methods employed for culturing pneumococci on solid media have resulted in slow and uncertain growth. This is particularly true when the isolation of pneumococci from a mixed culture is attempted. In routine sputum examination for pneumococci the injection of mice has been the most reliable method of isolation, especially when the pneumococci in the sputum are outnumbered by other organisms. However, the mouse method of culturing is beset with pitfalls. A certain type of pneumococcus for example may be isolated from a sputum due to the fact that the mouse may be more sensitive to one type of pneumococcus which is only acting in the role of a mouth organism than to another type of pneumococcus actually causing the pneumonia. Again, a mouse is more susceptible to pneumococci in general than to other pathogenic organisms, and it is quite possible to miss an organism other than pneumococcus which may be contributory to the cause of the pneumonia. Mixed infections are very common among infants, and often explain the poor response to specific therapy. The other objection to mouse culturing as a routine procedure is the expense and special attention involved.

To obviate these difficulties a much simpler routine laboratory procedure has been developed at this hospital which, in the hands of an experienced technician, can supplement and in most cases replace the mouse method of isolating pneumococci from sputum. This development was made possible by the observation of a stimulation of growth for all types of pneumococci in the presence of carbon dioxide. This "sputum plate" method does not entirely eliminate the mouse method of culturing, but provides an easy, reliable and fast method of growing pneumococci. At the same time the method encourages the growth of other pathogenic respiratory organisms such as *B. influenzæ* or *Streptococcus hæmolyticus.*

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LITERATURE.

Carbon dioxide in atmospheric concentration of 10 per cent. is generally recognized as stimulating the growth of *B. abortus*, gonococcus and meningococcus. Gladstone, Fildes and Richardson (1935), Valley and Rettger (1937) and Rockwell and Highberger (1927) have reported carbon dioxide a definite stimulant for all bacteria, particularly for recently isolated strains. Little attention has been paid to the possible relationship between pneumococci and carbon dioxide, although the luxuriant growth of pneumococci in lung parenchyma is suggestive. Rockwell (1923) isolated a strain of pneumococcus from the pus of a knee-joint which required carbon dioxide for its growth. Nye and Lamb (1936) advised the growing of all routine cultures in a jar in which the burning of a candle produced about 3 per cent. carbon dioxide, and cited the case of a type VIII pneumococcus, which on subculture from a blood broth media to blood-agar plate did not grow without carbon dioxide. Except for these brief references to pneumococcus and carbon dioxide apparently little further investigation has been carried out. After routine culturing in carbon dioxide of numerous strains of pneumococci from sputa and exudates, it is evident that the growth of pneumococci on blood-agar plates is definitely stimulated by the carbon dioxide. Only a practical application of this relationship is presented in this paper in that the more or less theoretical aspects of the subject will require further experience and more detailed experiment.

METHOD.

The sputum is collected from the nasopharynx in children or from the larynx in adults, using the aspiration method of obtaining sputum (Auger, 1939). This latter method ensures that the patient does not salivate instead of expectorate. The sputum is then washed in a Petri dish with about 10–20 c.c. of saline, which tends to remove mouth organisms and leave the purulent particles containing the likely pathogenic bacteria. A fresh blood-agar plate (not older than 18 hours) is then inoculated. This culture plate consists of 15 c.c. of 2 per cent. nutrient agar, to which is added 1 c.c. of human or sheep's blood. The purulent particle is well stirred in the saline and then picked out using a straight piece of platinum wire. The size of this particle depends on the appearance of the sputum on direct smear. If there are numerous organisms present then the particle must be correspondingly small and *vice versa*. The chief difficulty of the procedure lies in the selection of a suitable particle, because it ought to be no larger than 2 mm. in diameter. If there is any doubt about the particle being representative, then the procedure should be carried out in duplicate. This particle is then placed on the centre of the plate and is spread thinly with a glass spreader. The spreading must be done carefully, and the best method is to make two or three initial spreads across the plate in one direction. The spreader is then flamed, and the margins of the initial streak are then spread upon the rest of the plate. The glass spreader itself must not be too thin or it will cut the media and it must be held lightly while stroking. When one is experienced, half or third of a plate

is sufficient for one sputum culture. A piece of wet blotting-paper about 3 by 3 cm. is then placed on the inner surface of the lid of the Petri dish. The blotting-paper should be previously autoclaved and dipped into boiling water to prevent gross contamination of the plate.

The plate is then placed with the blood-agar side uppermost in the bottom of an air-tight container. In routine work ordinary 1400 c.c. tin cans with close-fitting lids are used, but for more accurate work glass or metal anaërobic jars are used. Carbon dioxide is then introduced into the vessel containing the Petri dish. The carbon dioxide is obtained from a commercial cylinder and is led off into a water burette (300 c.c. capacity), and finally introduced into the bottom of the container by means of a rubber tube past the partially opened lid. The actual amount of carbon dioxide introduced does not matter, since it is equally effective over a range of 0·5 to 25 per cent. This means that one should place a minimum of about 100 c.c. of carbon dioxide in a 1400-c.c. can to allow for leakage past the open lid. After the required amount of carbon dioxide has been introduced slowly into the bottom of the can the tube is removed and the lid is sealed with adhesive tape. The container must be air-tight, and is then incubated for 15 hours between 35–37° C. The container should be washed with warm water before being used again.

RESULTS AND DISCUSSION.

This " sputum plate " method depends on the marked stimulating effect of carbon dioxide in the presence of adequate moisture on the growth of pneumococci. It is also effective for *B. influenzæ*, *Streptococcus hæmolyticus*, staphylococcus and other pathogenic bacteria found in the respiratory tract. The differentiation of mouth organisms and *Streptococcus viridans* from pneumococcus is usually simple. The large, smooth, shiny, watery appearance of the pneumococcus growth makes an easy comparison with the smaller dull-surfaced colony of streptococcus. Greey's (1939) test for detecting streptococci from pneumococci by sprinkling dried bile over the colonies is particularly efficient when the colonies are grown in carbon dioxide. Thus a more representative or a better " cross section " of the flora in the sputum is obtained.

Ordinarily pneumococci when grown in the presence of other organisms, particularly staphylococci, are usually smothered unless numerous enough to assert themselves. But in the presence of carbon dioxide and extra moisture pneumococci grow as reliably and as rapidly as any other organism. Usually a maximum growth of pneumococci is possible in 15 hours with this method. This method of isolating pneumococci is also suitable for growing all strains of pneumococci, including the so-called anaërobic strains (Smith, 1936). In several instances it has been possible to identify two different types of pneumococci from a single sputum by careful examination of colony morphology.

The illustrations show the results of growing three blood-agar plates, each placed in a separate anaërobic jar containing 0·03 per cent. carbon dioxide (atmosphere), 0·5 and 25 per cent. carbon dioxide. Each plate was trisected

and spread similarly. The first segment received a particle of sputum containing type III pneumococci, the second segment a small loopful of cerebro-spinal fluid containing type XIX pneumococci, and the third segment a small loopful of a pleural fluid containing type I pneumococci. The photographs show a scanty growth from the sputum on the control plate. This growth consisted chiefly of staphylococci, but a luxuriant moist growth of type III pneumococci overgrowing the staphylococci on the plates was obtained with addition of carbon dioxide to the jar. The growths from the cerebro-spinal fluid and pleural fluid are similar, inasmuch as there is no visible growth in 15 hours with ordinary atmospheric conditions, but luxuriant growths of pneumococci were again obtained with addition of carbon dioxide.

The gross features of the colonies from the three types are remarkably similar. The colonies are pale green (human blood was used for the plates), watery, irregular in outline, and have tended to coalesce. Where the colonies were discrete, a slight umbilication was usually present. After standing the colonies flatten and shrink. Capsular swelling was unaffected by this rapid growth. These strains were chosen arbitrarily to show the universal stimulating power of carbon dioxide for pneumococci.

The stimulating effect of carbon dioxide on pneumococci is now being studied in detail, but it was felt that this practical application should be presented now since it has been found extremely useful in this laboratory during the past year. In routine pneumonia work the mouse inoculation method is limited to sputum which has failed to reveal a likely pathogen either on direct examination (direct smear and Neufeld method) or on the sputum plate. These failures, which in our experience with children amount to 5–10 per cent. of cases, are due to lack of suitable sputum.

SUMMARY.

1. A " sputum plate " technique for the isolation of pneumococci has been presented.

2. The stimulating effect of carbon dioxide on respiratory pathogens, particularly pneumococci, has been noted.

3. A maximum growth of pneumococci in 15 hours on blood-agar plates is possible with this method.

The author wishes to acknowledge the helpful suggestions of Dr. W. L. Holman, Professor of Bacteriology, University of Toronto.

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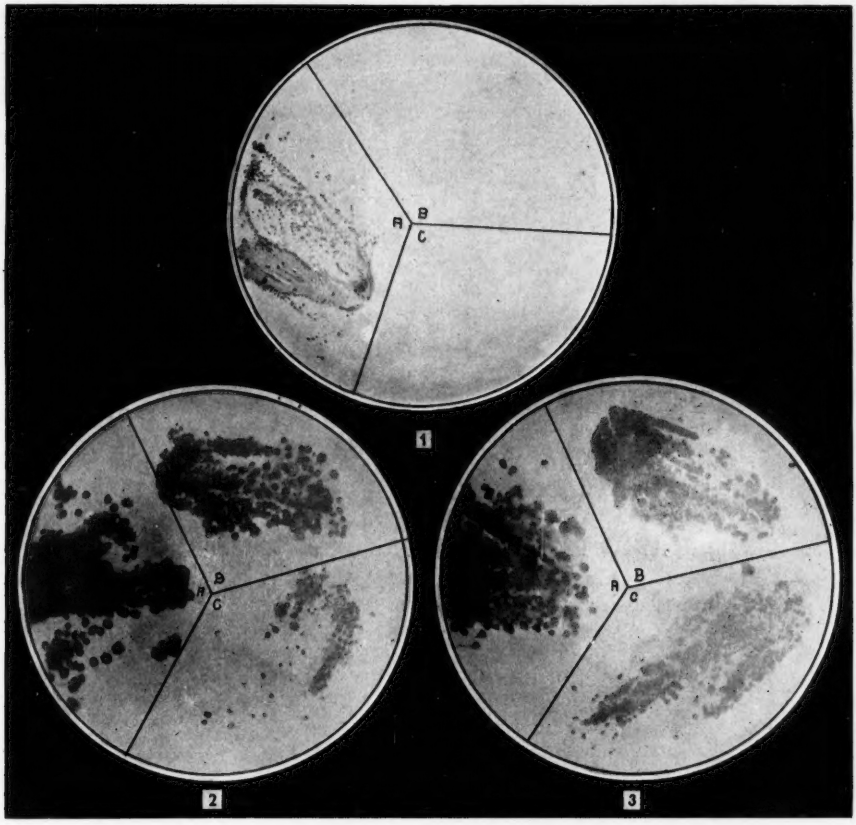

FIG. 1.—Photograph of 15-hour cultures of pneumococci on blood-agar plate grown in ordinary atmosphere which contains approximately 0·03 per cent. carbon dioxide. (A) Sputum Type III. (B) Cerebro-spinal fluid Type XIX. (C) Pleural fluid Type I. Note the scanty growth from the sputum and no visible growth from either the cerebro-spinal fluid or pleural fluid.

FIG. 2.—Photograph of 15-hour cultures of pneumococci on blood-agar plate grown in an atmosphere of 0·5 per cent. carbon dioxide. The source and position of cultures are the same as in Fig. 1. Note the luxuriant growths of pneumococci in all three segments.

FIG. 3.—Photograph of 15-hour cultures of pneumococci on blood-agar plate grown in an atmosphere of 25 per cent. carbon dioxide. The source and position of cultures are the same as before. Again note the luxuriant growth of pneumococci in all three segments.

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FACTORS INFLUENCING THE INFECTIVITY OF FOWL TUMOURS.

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IN two previous communications a number of sarcomata induced with tar in fowls were described (McIntosh, 1933; McIntosh and Selbie, 1939). Nine of the tumours were propagated in series, and five were repeatedly passed by means of Berkefeld filtrates. Four of the tumours could not be successfully filtered, although repeated attempts were made. In the present communication it will be shown that the filterability or non-filterability of a fowl tumour apparently depends to a great extent on the virulence of the tumour, and that in some cases the initial virulence of an apparently non-filterable tumour may be raised so that it can be filtered. Experiments will also be described showing that the virulence of tumour suspensions can be increased by proteolytic digestion and by repeated freezing and thawing.

RELATION OF FILTERABILITY TO VIRULENCE.

The relative virulence of our tar tumours is estimated in Table I by comparing the average interval between inoculation and the time that the tumour is first palpated, the average interval between passages and the percentage of positive suspension inoculations. Only tumours that could be maintained by inoculations of dilute suspensions are included in this table, so that tumour 10, which could be passaged only with minced tumour tissue, and the later passages of Tumour 7, when it could no longer be transmitted by suspensions, are omitted. The tumours are arranged in order of virulence, and they fall into two sharply defined groups of filterable and non-filterable tumours. The filterable tumours are in all respects much more virulent than the non-filterable tumours. It would appear, therefore, that the filterability of a fowl tumour depends on its virulence or, conversely, the virulence of a fowl tumour is an expression of the virulence or activity of the virus.

Most of the filterable tumours, however, were relatively avirulent at the outset, as is shown in Fig. 1, which is a chart of the first five passages of Tumour 9. The proportion of successful suspension inoculations was only 2 out of 14 in the first passage, but increased to 6 out of 6 in the fourth passage.

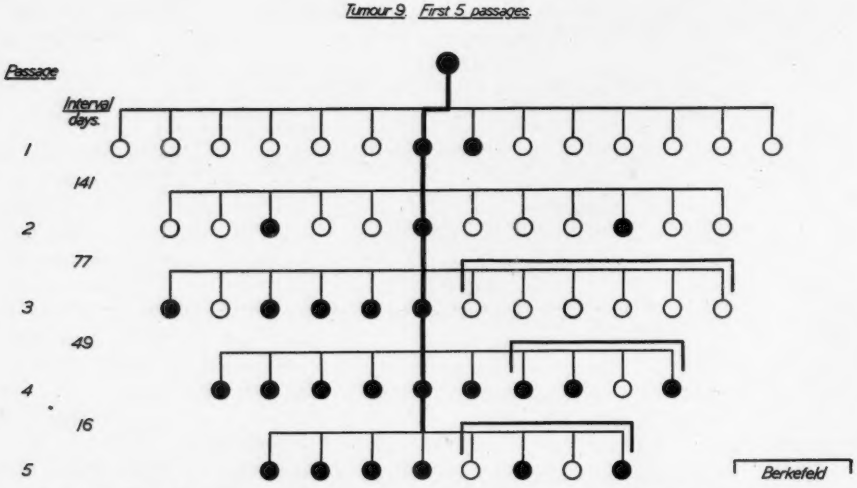

FIG. 1.—First five passages of Tumour 9, showing increasing virulence with successive passages and successful filtration in 4th passage after failure in 3rd passage.

TABLE I.—*Virulence of Filterable and Non-filterable Tar-induced Fowl Tumours.*

Tumour.	Average time to produce palpable tumour (days).	Average interval between passages (days).	Inoculations of suspensions, per cent. positive.
Filterable :			
No. 5	15	25	97
,, 9	15	25	92
,, 2	20	33	85
,, 3	22	51	62
,, 8	33	62	69
Non-filterable :			
No. 4	60	85	27
,, 1	75	82	14
,, 7	96	121	15

The tumours also appeared earlier and grew more rapidly, as is indicated by the decreasing intervals between passages. At the third passage the tumour was non-filterable, but it became filterable at the fourth passage. Tumour 9, therefore, at first a relatively avirulent non-filterable tumour, became filterable as its virulence increased. The other filterable tumours behaved in a similar manner, but the virulence of the remaining tumours did not increase sufficiently and they continued to be non-filterable tumours.

Apart from the use of young tumours for passage and propagation in a susceptible strain of fowls, we considered that the great increase in virulence of our fowl tumours as shown in Fig. 1 was largely due to the use of dilute

suspensions, particularly after treatment with trypsin. An investigation was therefore made on the action of trypsin and on certain other agents producing a similar effect.

THE ACTION OF TRYPSIN.

Glycerine Extract of Pancreas.

Examples of the effect of a glycerine extract of pancreas on two filterable and two non-filterable chemically-induced fowl tumours are shown in Table II. Tumour suspensions, prepared by grinding the tumour with sand and adding saline to make a 2·5 per cent. tumour suspension, were treated with 5·0 per cent. liquor trypsin co. (Allen & Hanbury) for 2½ hours at room temperature. Suspensions of all four tumours are increased in virulence by trypsin in the same way as was described in the Rous Sarcoma No. 1 by Baker and McIntosh (1927). On several occasions (*e. g.* Tumour 7) tumours were found only after inoculations of trypsinized suspensions, so that propagation of the tumour with dilute suspensions would have failed if trypsin had not been used. An example is also given of the increased virulence of Berkefeld filtrates of trypsin-treated suspensions (Tumour 9, fourth passage). Although this enhancing effect of trypsin is the general rule, there have been occasional exceptions. The Sheffield dibenzanthracene tumour (Mellanby, 1934) usually responds favourably to trypsin, but on some occasions trypsin-treated suspensions have been less virulent than control suspensions. On the other hand, suspensions of the tar-induced tumour GRCH/13 (Peacock, 1935) are usually less active after trypsin treatment, but untreated suspensions of this tumour are, in any case, generally non-infective. These variations in the action of trypsin on fowl tumours may be expressions of biological differences in the tumours. On the other hand, a slight lowering of infectivity after treatment with liquor trypsin co. has occasionally been noted in the Rous sarcoma, as is shown in Table IV, where crystalline trypsin produced an increase in infectivity. Some of the variations noted may therefore be due to the occasional presence of some impurity in liquor trypsin co.

TABLE II.—*Effect of Liquor Trypsin Co. on Chemically-induced Fowl Tumours.*

Tumour 7. *8th passage. Expt.* 95/36.

Fowl No.:	282.		283.		284.		285.		286.		287.	
	O.	T.	O.	T.	O.	T.	O.	T.	O.	T.	O.	T.
72 days	..	..	−	−	−	+	..	..	−	+	−	+
118 ,,	..	..	−	−	−	+++	..	..	−	+++	−	+
Autopsy	−	−	−	−	−	+++	−	−	−	+++	−	+
(days)	16		163		118		19		133		120	

Tumour 8. *5th passage. Expt.* 77/37.

Fowl No.:	137.		138.		139.		140.		141.		142.	
	O.	T.	O.	T.	O.	T.	O.	T.	O.	T.	O.	T.
21 days	−	+	−	+	−	−	−	−	−	−	−	−
28 ,,	−	+	−	+	−	+	−	−	−	−	−	+
Autopsy	−	+	+	+	−	+	−	−	−	−	+	++
(days)	33		73		70		34		126		36	

TABLE II.—(*cont.*).

Tumour 9. 3*rd passage.* *Expt.* 152/36.

Fowl No.:	482.		483.		484.		485.		486.		487.	
	O.	T.	O.	T.	O.	T.	O.	T.	O.	T.	O.	T.
22 days	−	−	−	−	−	+	−	+	−	+	−	−
34 ,,	−	+	−	−	−	++	−	+	..	..	+	−
Autopsy (days)	− 49	+	− 79	−	− 34	++	− 50	++	− 30	++	+ 49	−

4*th passage.* *Berkefeld filtrates.* *Expt.* 207/36.

Fowl No.:	685.		686.		687.		688.	
	O.	T.	O.	T.	O.	T.	O.	T.
16 days	−	−	−	−	−	+	−	++
30 ,,	−	−	−	+	..	..	..	..
Autopsy (days)	− 74	−	− 44	+	+ 30	++	− 28	+++

Sheffield Dibenzanthracene Tumour. *Expt.* 86/36.

Fowl No.:	255.		256.		257.	
	O.	T.	O.	T.	O.	T.
14 days	+	++	+	+++	+	+++
Autopsy (days)	+ 17	++	+ 22	+++	+ 21	++

O = untreated tumour suspension.
T = tumour suspension treated with 5·0 per cent. liquor trypsin co. for 2½ hours at room temperature.

Dried and Crystalline Trypsin.

In Tables III and IV it is shown that dried trypsin (B.D.H.) and crystalline trypsin (kindly supplied by Dr. A. Pollard, Courtauld Institute of Biochemistry) are even more active than liquor trypsin co. in increasing the infectivity of suspensions of the Rous sarcoma. In Table V various fractions of an aqueous extract of dried trypsin are compared. The proteolytic activity of the fractions was tested on calcified milk, and it was found that fraction C was equal in activity to the original aqueous extract A, and that fractions B and D were almost entirely inactive. Table V shows that the activity of the fractions in increasing tumour infectivity corresponds to their proteolytic activity.

TABLE III.—*Effect of Dried Trypsin on the Rous Sarcoma.*

Expt. 133/35.

	Suspensions.					Berkefeld filtrates.			
Fowl No.:	311.	312.	313.	314.	Fowl No.:	307.	308.	309.	310.
Untreated	−	−	−	++	5·0 per cent. liq. trypsin co. (pH6)	−	+	+	+
Adjusted to pH6	−	−	−	−	1·0 per cent. dried trypsin (pH6)	−	+++	+++	+++
Day of autopsy	66	66	61	46	Day of autopsy	66	27	33	37

TABLE IV.—*Effect of Crystalline Trypsin on the Rous Sarcoma.*

Expt. 264/38. *Berkefeld filtrates.*

	Neat.				1/10.				1/100.			
Fowl No.:	435.	436.	437.	438.	439.	440.	441.	442.	443.	444.	445.	446.
Untreated	++	−	++	++	+	+	++	+	−	++	−	++
0·5 per cent. crystalline trypsin	+++	−	+++	++	++	+++	+++	+++	−	+++	−	++
5·0 per cent. liquor trypsin co.	++	−	++	++	−	+	+	+	−	−	−	−
Day of autopsy	21	9	20	20	17	18	18	18	31	33	9	31

TABLE V.—*Effect of Fractions of Dried Trypsin on Rous Sarcoma.*

Expt. 151/38. *Berkefeld filtrates.*

	Neat.			1/10.			1/100.		
Fowl No.:	220.	221.	222.	223.	224.	225.	226.	227.	228.
Untreated	..	+	−	..	+	−	..	−	+
A	++	..	+	−	..	++	−	..	++
B	−	−	−	−	+	−	−	−	−
C	++	+	+	−	++	−	−	−	++
D	+	+	..	−	+	..	−	−	..
Day of autopsy	21	23	16	3	41	41	41	18	22

Suspensions A, B, C, D were treated for 2½ hours with 1·0 per cent. of:—A—4·0 per cent. aqueous extract of dried trypsin (B.D.H.); B—A after denaturation of proteins by heat; C—A treated with $NaHCO_3$; D—proteins of A precipitated with $(NH_4)_2SO_4$ and redissolved in water.

Tests for precipitation of casein in calcified milk showed equal activity in A and C and practically none in B and D.

THE ACTION OF PEPSIN.

The effect of dried pepsin (B.D.H.) was tested on a suspension of Rous sarcoma at three different pH values. The amount of pepsin used (0·08 per cent.) was equivalent in proteolytic activity to 5·0 per cent. liquor trypsin co., as determined on calcified milk. The result of the experiment (Table VI) indicates that pepsin has an action similar to trypsin, for in the fowls inoculated with 1/10 and 1/100 dilutions tumours were produced only with pepsin-treated inocula. There is, however, no apparent difference in the action of pepsin at different pH values, as in trypsin, which is most active, in this respect, in an acid medium (Baker and McIntosh, 1927). In another experiment, done in a precisely similar manner with the Rous sarcoma, when it was passing through a phase of very low infectivity, only two tumours were found, and these were produced by inoculations of undiluted filtrate of a suspension treated with pepsin at pH 6.

TABLE VI.—*Effect of Pepsin on the Rous Sarcoma.*

Expt. 35/39. *Berkefeld filtrates.* 0·08 *per cent. Dried Pepsin (B.D.H.).*

	Neat.				1/10.				1/100.			
Fowl No.:	101.	102.	103.	104.	105.	106.	107.	108.	109.	110.	111.	112.
Untreated	++	+	+	+	−	−	−	−	−	−	−	−
Pepsin pH 6	++	++	+	−	+	−	−	+	−	−	−	−
,, pH 7	+	+++	+++	++	−	++	−	−	−	−	−	−
,, pH 8	+	+	−	−	−	+	−	+	−	−	+++	−
Day of autopsy	27	33	44	46	39	42	46	37	46	46	44	46

AUTOLYTIC ACTION.

Several passages of Tumour 7 were made from tumour tissue that had been kept in the refrigerator (0°–4° C.) for 24 hours. These experiments are shown graphically in Fig. 3 of our previous communication (McIntosh and Selbie, 1939). The average yield of tumours from suspensions of tissue kept in the refrigerator was somewhat greater than from suspensions of fresh tissue. An example is shown in Table VII, where tumours were produced only from tissue that had been kept for 24 hours. We are not in a position to say whether this finding applies to other fowl tumours, for it was tried with Tumour 7 only because of the great difficulty experienced in the propagation of this tumour.

TABLE VII.—*Effect of Autolysis on Tumour 7.*

4th passage. Expt. 69/35. *Tumour suspension.*

	Fresh.								Refrigerator overnight.							
Fowl. No. :	140.		141.		142.		143.		144.		145.		146.		147.	
	O.	T.	O.	T.	O.	T.	O.	T.	O.	T.	O.	T.	O.	T.	O.	T.
21 days .	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–
33 ,, .	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++	+
Autopsy .	–	–	–	–	–	–	–	–	–	–	–	–	+++	+++	++	++
(days) .	448		448		448		448		108		100		84		49	

O, T : as in Table II.

THE ACTION OF REPEATED FREEZING AND THAWING.

Technique.

The tumour is finely minced and sealed in 5·0 c.c. ampoules, each containing about 1·0 g. of minced tumour. The ampoules are then rapidly frozen by complete immersion in solid CO_2 and ether, which is kept at a temperature of −79° C. by maintaining an excess of solid CO_2. After each freezing the tissues are rapidly thawed in a water-bath at 37° C. The tissues are frozen and thawed three times, the first freezing lasting 45 minutes, and the second and third 10 minutes each.

Results.

Examples of such experiments with the Rous sarcoma and two filterable tar-induced tumours are shown in Tables VIII and IX. Berkefeld filtrates prepared from suspensions of frozen and thawn tumour are much more active than filtrates of untreated tumour. The action of freezing is also compared with the action of trypsin in two tar-induced tumours (Table IX). The infectivity of the two tumour filtrates is increased by both trypsin and freezing, but it is remarkable that a greater effect is produced by freezing in Tumour 5, whereas the greater effect is produced by trypsin in Tumour 9. Suspensions of frozen and thawn non-filterable fowl tumours, however, have in our hands been non-infective, and this has also been our experience with the transplantable mouse tumours sarcoma 37S (Imperial Cancer Research Fund), Krebs

leukosis (Kaalund-Jørgensen, 1936), and the Ehrlich carcinoma both as a subcutaneous tumour and in its fluid ascitic form (Collier, 1934), although it has been shown by Breedis and Furth (1938) and others that cells may survive similar, though not identical treatment.

TABLE VIII.—*Effect of Freezing on Rous Sarcoma.*

Expt. 281/38. *Berkefeld filtrates.*

	Neat.		1/10.		1/100.	
Fowl No.:	480.	481.	482.	483.	484.	485.
Untreated	−	−	−	++	−	−
Frozen	−	++	+	+++	−	++
Day of autopsy	9	24	44	31	40	29

TABLE IX.—*Effect of Freezing and Trypsin on Tumours 5 and 9.*

Tumour 5. *Expt.* 408/38. *Berkefeld filtrates.*

	Neat.				1/10.			
Fowl No.:	874.	875.	876.	877.	878.	879.	880.	881.
A. Untreated suspension	−	+	+	+	−	−	−	−
B. Suspension A trypsinized	−	+	+	++	−	−	−	−
C. Suspension of frozen tumour	++	++	++	++	−	−	−	−
D. Suspension C trypsinized	+++	+++	+++	+++	−	−	−	−
Day of autopsy	29	26	14	20	43	29	43	43

Tumour 9. *Expt.* 411/38. *Berkefeld filtrates.*

	Neat.				1/10.			
Fowl No.:	888.	889.	890.	891.	892.	893.	894.	895.
A. Untreated suspension	+	++	+	+	−	−	−	−
B. Suspension A trypsinized	++	+++	++	+++	+	−	+++	−
C. Suspension of frozen tumour	+	−	++	++	+	−	++	−
D. Suspension C trypsinized	−	++	−	−	−	−	+	−
Day of autopsy	32	22	34	34	27	42	28	4

DISCUSSION.

Baker and McIntosh (1927) have shown that increased infectivity of tumour suspensions after treatment with liquor trypsin co. is not essentially due to the reaction of the medium (pH 6) which, indeed, by itself causes a fall in infectivity. Several experiments have also shown that glycerine, of which there is a large amount in this preparation, has no action in either direction. The experiments with other preparations of trypsin that we have just described would further indicate that the enhancing effect is brought about entirely by the proteolytic action of the trypsin. The experiments with pepsin and the effect of autolysis on Tumour 7 also support the view that the enhancing effect is due to proteolytic action.

Claude (1935, 1939), following on the experiments of Murphy and Sturm (1932), showed that after certain procedures the virulence of chicken-tumour extracts could be increased apparently by the removal of a constituent. This constituent he considered to be an inhibitory factor, and it could be removed by dialysis and by centrifugalization and washing the sediment. In his most recent experiments Claude states that he was able to destroy this inhibitory

substance by trypsin action. But the fact that our freezing and thawing experiments show that another entirely different method of breaking up tumour tissue produces a similar effect indicates that the process is not so simple as Claude has suggested.

The increased infectivity of suspensions of the filterable fowl tumours after digestion or freezing can be well understood, for more virus is liberated by the breaking up of the tissue fragments, and is thus made more available for attacking normal tissues. In non-filterable tumours, however, that is to say, in tumours where virus plays no part in transmission, there could be no such effect. This has been our experience with the transplantable mouse tumours, in that they are unaffected by trypsin, and cannot be transplanted after being subjected to our method of repeated freezing and thawing. The non-filterable fowl tumours, however, fall into an intermediate group. In the respect that suspensions of repeatedly frozen and thawn tumour tissue fail to produce tumours, they resemble the mouse tumours. On the other hand, they are like the filterable tumours in that suspensions are usually increased in infectivity by the action of trypsin.

Another feature in the behaviour of these chemically-induced fowl tumours, which has been referred to in our previous communication (McIntosh and Selbie, 1939), is the increase in virulence of tumour suspensions after repeated passages with suspensions (Fig. 1). We suggested that by repeated passage under conditions unfavourable for cellular transmission the virus is trained to take a more active part until finally, in many cases, the tumour can be transmitted by means of Berkefeld filtrates. This training probably takes the form of a selection of a particular strain of more active virus. Evidence that virus is present in non-filterable chemically-induced fowl tumours has also been given by Andrewes (1936) and Foulds (1937), and it is probable that in these tumours the virus is unable to attack normal cells, or is too large to pass through a Berkefeld filter.

It is well known that filterable tumours sometimes go through a phase of non-filterability such as the Rous sarcoma No. 1 (Gye and Andrewes, 1926), and this may be due to the presence of an inhibitory substance in the filtrates or some temporary modification of the virus. Such an explanation might account for the non-filterability of some of the fowl sarcomata that cannot be sufficiently increased in virulence to provide active filtrates.

It would appear, however, that the current method of distinguishing virus tumours from non-virus tumours by the success or failure of Berkefeld filtrates is too exacting. Some of the larger viruses pass through Berkefeld filters only with the greatest difficulty. We have found that suspensions of vaccinia are greatly reduced in infectivity by passage through a Berkefeld filter. In two recent experiments Berkefeld filtrates of vaccinia had no infectivity, whereas the original suspensions had titres of 10^5 and 10^6. We have obtained on several occasions successful passages of the Sheffield dibenzanthracene tumour and the tar tumour GRCH/13 by means of standard sand and paper-pulp filtrates, but so far all attempts to pass these tumours through Berkefeld filters have failed. It is therefore a possibility that the virus of some fowl tumours cannot pass through a Berkefeld filter because of either its size or its physical state.

SUMMARY.

The filterability of chemically-induced fowl tumours depends to a great extent on the virulence of the tumours and, conversely, the virulence of such a tumour depends on the activity of the virus.

The virulence of such a tumour, and therefore the activity of the virus, can be increased by methods designed to discourage cellular transmission.

Suspensions of filterable and most non-filterable fowl tumours can be increased in infectivity by proteolytic digestion.

Repeated freezing and thawing by the technique described increases the infectivity of suspensions of the filterable fowl tumours, but annuls the infectivity of the non-filterable tumours.

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TISSUE CHANGES IN ASCORBIC ACID DEFICIENT GUINEA-PIGS.

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THE pathological changes found in scurvy have recently been reviewed by Dalldorf (1938) and an extensive summary of the literature is consequently unnecessary in this paper. Abnormalities in the teeth, bones, heart and other tissues have been described and two general explanations have been offered. Much emphasis has been placed on the theory advanced by Wolbach and Howe (1926) and supported by Mazoue (1937) that vitamin C is concerned directly with the physical nature of intercellular material, and that scorbutic changes are the result of the failure of specialized cells to secrete some substance which normally would produce jellying of collagenous material. On the other

hand, extensive experiments conducted by Höjer and Westin (1925) and Fish and Harris (1934) led to the conclusion that vitamin C deficiency causes a degeneration of cells with specialized functions, such as odontoblasts, osteoblasts, etc., and that all other structural effects are secondary. This view is also accepted by Ham and Elliott (1938), who have made an accurate study of bone and cartilage lesions in protracted moderate scurvy. These authors have also presented a critical discussion of the jellation theory.

However, much of the reported work has been rendered obscure by the use of complex sources of vitamin C and the pure vitamin has been employed only in a few instances. In no case has the inanition accompanying scurvy been taken into consideration. For this reason the following experiments were undertaken, using the paired feeding technique, which eliminates inanition as a variable factor, and pure l-ascorbic acid as the source of vitamin C.

METHOD.

The method of paired feeding employed by us has already been described in detail (McHenry, Reedman and Sheppard, 1938). It consists in maintaining young guinea-pigs, 200–230 g. in weight, for a week on the basal diet plus 10 mg. of ascorbic acid daily in order to saturate them with vitamin C and to accustom them to changes in diet and housing. They are then divided into three groups equivalent with respect to weight, sex and rate of weight gain during the previous week. Group I is then fed basal diet *ad libitum* with no supplement. Group II animals are allowed to eat only the amount consumed by paired mates in Group I, and are given in addition 5 mg. of ascorbic acid daily by mouth. Group III serves as a normal control and is allowed to eat *ad libitum* while receiving 5 mg. of ascorbic acid per day. Deficient animals receiving the basal diet die in 21–28 days.

In order to have complete groups for comparison with paired mates, the experiments reported here were generally terminated on the 21st day. Previous work indicated that at this time deficient animals showed external symptoms of acute deficiency. In some experiments groups of animals have been studied after 10 days' deficiency and, in other cases, after curative periods of various lengths following the 21-day deficient period. Animals were stunned and bled out. Sections from the various organs were immediately dissected out and placed in 10 per cent. formol saline. These were embedded in paraffin and subsequently stained usually with hæmatoxylin and eosin. In some cases Mallory's connective-tissue stain after Zenker's fixation was used. Calcified tissues were decalcified in 5 per cent. nitric acid.

RESULTS.

The histological findings are given for the different tissues studied.

Thyroid: Nearly all these were in a resting state with normal colloid. The follicular cells were of a low cuboidal or flattened type. No histological evidence of increased thyroid activity in the scorbutic group was obtained, although previous work from this laboratory demonstrated an increased metabolism in such animals (Fidlar, Sheppard and McHenry, 1939).

Parathyroids, lymph-glands, trachea, œsophagus and carotid arteries: These showed no abnormality or difference between the groups.

Adrenals: No significant differences were found between groups. Some fine vacuoles indicating that lipoid material had been present were observed in nearly all sections of adrenal cortex. One or two animals showed vascular congestion at the cortico-medullary junction.

Heart: No significant difference between the groups was obtained in the heart muscle, coronary arteries and veins, nor in the heart valves. The majority of the hearts showed small areas of mild degeneration, as evidenced by poor staining or shrunken fibres or tiny collections of wandering cells near the vessels. Such changes were not characteristic of any one group. The heart valves appeared normal, and changes as reported by Rinehart and Mettier (1934) were not found to be characteristic of the scorbutic group.

Liver: These were found to vary from strictly normal to ones showing mild degeneration as indicated by a fine vacuolization and thinning out of the cytoplasm, particularly in the central vein areas. Nearly all the scorbutic group had normal livers, whereas these minor degenerative changes were found mostly in the ascorbic acid-fed groups.

Intestines: Sections from the duodenum and jejunum of most of the animals were studied. These all presented an essentially normal appearance, both in the gross and microscopically. The epithelial cells of the villi, however, were more vacuolated in the groups fed ascorbic acid than in the scorbutic group. This finding requires further study before it can be stated to be significant. Other unpublished observations made by us indicate that ascorbic acid has an effect upon fat absorption.

Teeth: The teeth of the guinea-pig show persistent growth throughout life. At any one time, therefore, by sectioning longitudinally, all the various stages from initial development through maturity to senility in the life-history of the tooth can be seen. The advantage of longitudinal sections has been emphasized by Fish and Harris; cross-sections may give misleading impressions.

Fig. 1 shows a molar tooth from a guinea-pig of Group III (ascorbic acid plus basal diet *ad libitum*). In the root one can pick out the beginning of the odontoblastic layer responsible for the formation of dentine, and the ameloblastic layer responsible for enamel formation. As one examines these layers distal from the root it is evident that the odontoblasts are in an orderly position, and the dentine they exude is connected by a fine dentinal tubule or fibril with the cell proper. The older, *i. e.* more distal the odontoblast, the more dentine has been formed until maturity is reached at a point *A*, where the dentine is at its maximal width. The ameloblasts evidence a similar growth with formation of enamel. This has an insoluble matrix for a certain distance distal from the root, and beyond this point is dissolved by the decalcification procedure. Distal from point *A* the odontoblasts begin to show senile deterioration, lose their fibrils, become disorganized, shrunken, and may show vacuoles between themselves and the primary dentine. Their activity is now over and the pulp behind them narrows and finally ends. The remaining odontoblasts become engulfed in the calcific scar or osteodentine, and certain of them persist as islands in the centre of the tooth even to the biting surface. The pulp space

is wide at the bottom and narrows to a point distally. It is made up of a reticulum of fibroblast-like cells, in which are some blood-vessels.

If one examines Fig. 2 a similar normal appearance is found in all the tooth structures. No evidence of a defect due to the inanition factor of scurvy is present.

Fig. 3 shows a typical tooth from the scorbutic group. In these animals the odontoblasts and ameloblasts begin in a normal manner, but the dentine they form is altered in appearance. Hence, up the inner side of the original dentine a calcified (blue staining) line of scar-tissue (abnormal dentine) is laid down. This seems to cut off the dentinal tubules and marks the beginning of scurvy. The odontoblasts lose their regularity of formation and form a wavy border or serrated edge. The dentine formed after this time is laid down inside the original dentine but outside the odontoblastic layer, and lacks the tubular appearance of normal dentine. As the period of scurvy lengthens proportionally, more of the calcific scar-tissue occupies the area of the tooth ordinarily filled by the normal dentine. The odontoblasts now irregularly arranged become partially engulfed in this scar-tissue. The pulp-cavity is filled with normal pulp at the bottom of the tooth, but in the upper regions tends to persist as a potential cavity. The pulp cavities do not show increased vascularity, hæmorrhages, or œdema. The inter-tooth structures show no abnormality peculiar to either scurvy or inanition.

Sections close to the first molar were made through the lower incisor teeth from all the animals. These corroborated the findings in the molar teeth. Unless one is careful to cut a section close to the incisor root, the appearance in cross-section may not be representative of the growing part of the tooth. Fig. 4 shows the regular odontoblastic layer with uniform type of dentine, and a normal pulp-cavity. The scorbutic picture (Fig. 5) shows the irregular indentations of the odontoblastic and dentine layers.

Ribs : The costochondral junctions presented a normal appearance in Groups II and III (Fig. 6). The scorbutic group showed a normal cartilage. but a widened and/or fractured junction of the cartilage and bone (Fig. 7) The cartilage side of the zone of ossification appeared normal, but in the region where normally the primitive bone-marrow meets the cartilage layer there was a mass of mesenchymal cells. In some ribs a considerable amount of partially dissolved cartilage columns remained, particularly near the outer edges of the rib. In others there was only this mass of osteogenic tissue extending for some distance from the cartilage before the normal bone-marrow was met. Some hyaline or fibrinoid degenerative areas were seen at the edges of the costochondral junction. The compact bone was usually fractured or deficient in the growing area. The muscle attachments at this area frequently showed some hæmorrhages between the muscle-fibres. At the 21-day interval practically no ossifying trabeculæ were seen.

Knee-joints : The findings in regard to the joints may be dealt with under three headings—the diaphysis, the epiphysis and the periarticular structures.

Diaphysis : Growth of the bones, normally, in these animals is at two sites, the epiphysial plate and the periosteum. In the epiphysial plate the cartilage-cells proliferate, enlarge and then degenerate; the spaces occupied by the cartilage-cells become filled with osteogenic tissue. The resulting

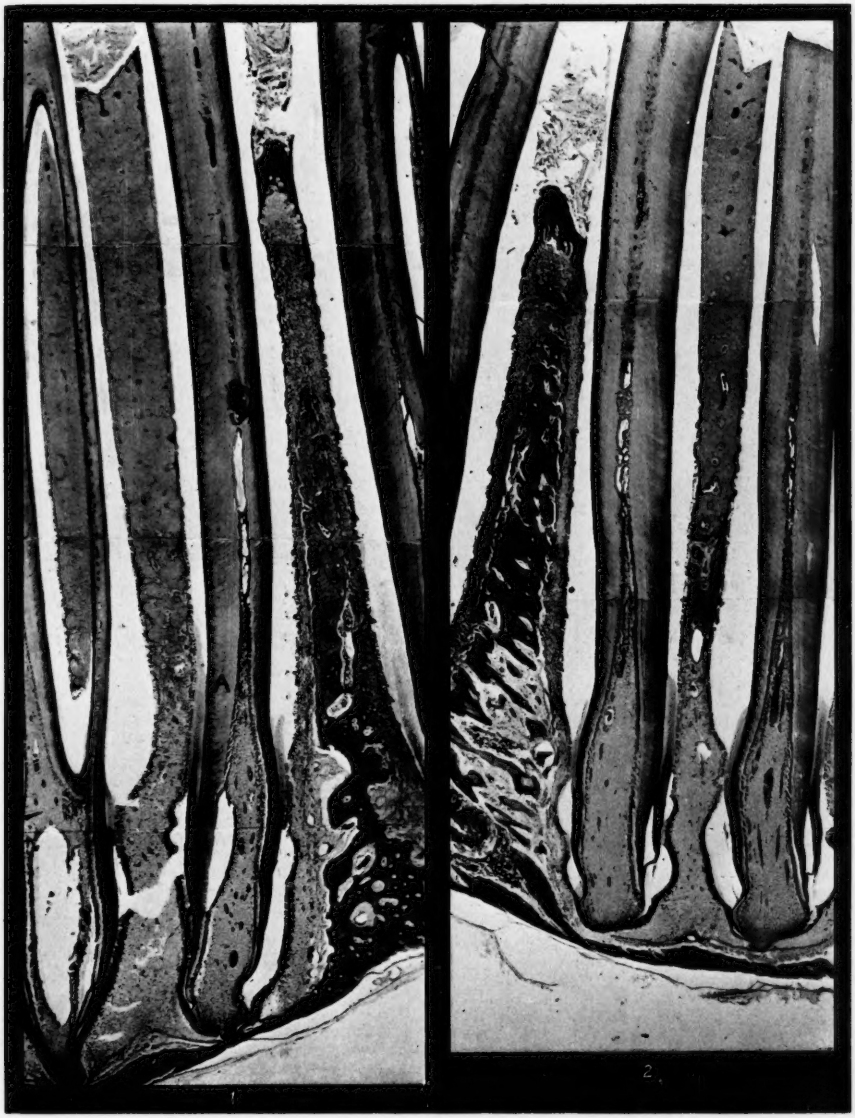

FIG. 1.—Molar teeth of guinea-pig fed basal diet *ad lib.* plus ascorbic acid (normal).

FIG. 2.—Molar teeth of guinea-pig given ascorbic acid, but paired in food intake with deficient animal.

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Fig. 3.—Molar teeth of guinea-pig deficient in ascorbic acid.

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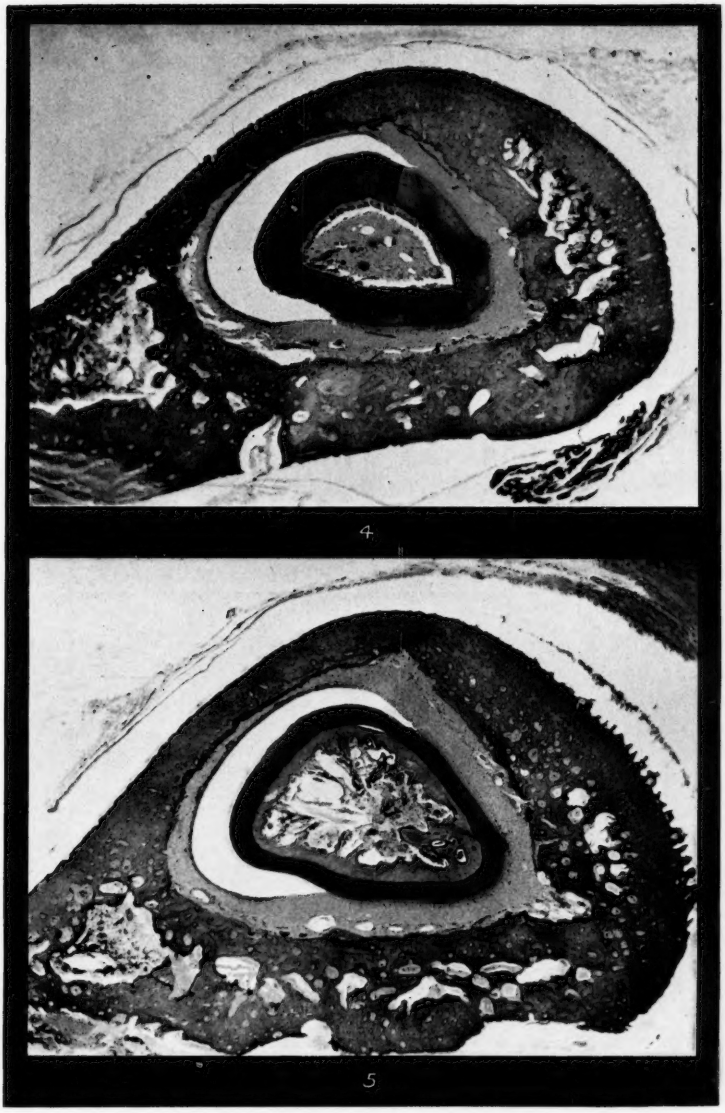

FIG. 4.—Section of lower incisor tooth of normal guinea-pig.

FIG. 5.—Section of lower incisor tooth of guinea-pig deficient in ascorbic acid.

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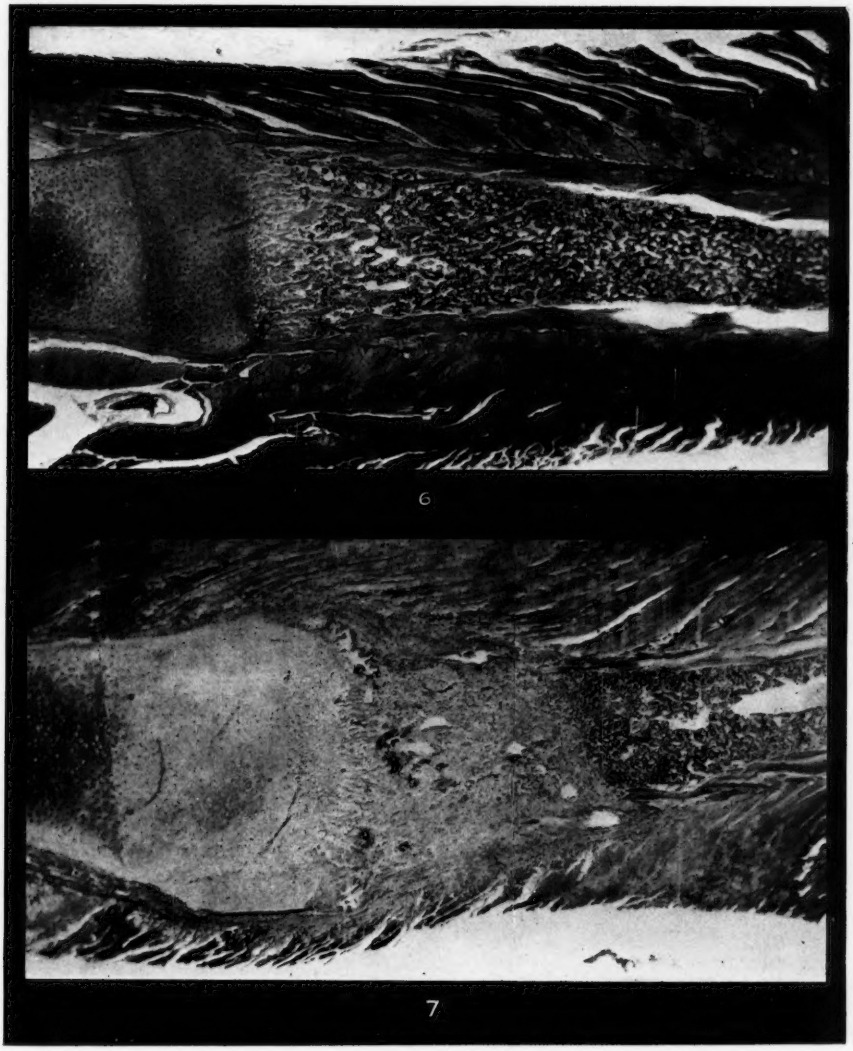

FIG. 6.—Costochondral junction of paired fed guinea-pig receiving ascorbic acid similar to animal fed *ad lib.*

FIG. 7.—Costochondral junction of guinea-pig deficient in ascorbic acid.

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picture is one of columns of old calcified cartilage separated by early marrow spaces. This zone of calcified cartilage is replaced by bony trabeculæ as the osteoblasts secrete or exude their bony matrix around the rapidly disappearing columns of calcified cartilage. This zone of new trabeculæ is always a more prominent feature at the periphery of the diaphysis and epiphysis than in the exact centre of the epiphysial plate. In older portions of the growth, *i. e.* towards the centre of the shaft and away from the epiphysial plate, the trabeculæ are absorbed and only persist close to the inner edge of the compact bone of the shaft. Growth at the outer edge of the shaft comes from osteoblasts derived from the osteogenic cells of the periosteum without going through the cartilage-cell stage in formation. Careful examination of the knee-joints of the experimental animals revealed no significant difference in the width of the epiphysial plate nor in the number of cartilage-cells in the columns of calcifying cartilage, either as a result of a deficiency of ascorbic acid or of inanition. Our observations are that the change in bone-formation due to ascorbic acid deficiency begins in the zone of ossification. The calcified cartilage columns seem to be resorbed in a normal manner, but the mesenchymal cells and/or osteoblasts accumulate in large numbers and do not secrete or exude the bony matrix. The result is that in 21 days nearly all the bones show an irregular fibrous network as a cap to the marrow cavity and below the zone of calcified cartilage columns. This massing of mesenchymal cells or deficient osteoblasts is often particularly dense at the periphery of the epiphysial plate, and even extends down on the outer side of the compact bone-shaft. Frequently in these areas a fair-sized acidophilic hyaline or fibrinoid degenerated spot was present. In many instances the upper end of the diaphysis appeared to be dislocated or fractured, and in these instances usually a massing of mesenchymal cells accompanied by hæmorrhages was evident at the outer edge of the epiphysial disc and next to the shaft of the bone. None of these abnormal changes was noted in the paired fed or *ad libitum* fed groups of animals, indicating that they are caused by a deficiency of ascorbic acid.

Epiphysis : Ham and Elliott have called attention to changes in the epiphysis in protracted moderate scurvy, notably an apparent deficiency in the bone support for the articular cartilage. In the animals examined in this study these differences between the scorbutic and normal groups were also noted. In general the articular cartilages appeared normal. No evidence of a " *Gerustmark* " or scorbutic change, as seen in the diaphysis, was noted in any of the epiphyses.

Periarticular structures : These showed some thickening and marked infiltration of red blood-cells and fibroblasts in the scorbutic group. The red blood-cells were mostly distributed as if by diapedesis between the muscle and tendon attachments around the joint, particularly at the epiphysial plate. No evidence of bleeding into the joint cavity and only rarely any mass of blood as from a ruptured vessel was found.

X-ray studies : Pictures of the costochondral junctions, knee-joints and the teeth revealed no particular difference in the calcification in any of the three groups. In the animals suffering from scurvy, however, many of the ribs showed marked swelling or flaring out at the costochondral junction. In the

histological study these swollen ribs were found to be ones showing evidence of dislocation or fracture. The line of calcification in the scorbutic group did not appear to be denser or thicker than in the other control groups.

DISCUSSION.

Our findings with regard to bone structure are in partial agreement with those of many other investigators, notably Höjer (1924), Aschoff and Koch (1919), in that we found that zones of normal bone-formation were replaced in the scorbutic group by a narrow framework (*Gerustmark*) composed of mesenchymal cells or deficient osteoblasts. Ham and Elliott did not obtain this change in their animals on a diet moderately deficient in ascorbic acid. We are in agreement with Ham and Elliott in that we did not, as a rule, find accumulations of calcified cartilage "lattice" in the scorbutic group as reported by Park *et al.* (1935), but in our experiments the epiphysial plate is not dormant in its centre or elsewhere. Both these groups of investigators, however, studied protracted moderate scurvy.

We feel the chief lesion is one that has to do with the differentiation of mesenchymal cells to osteoblasts, or with the stimulation of the osteoblasts to secrete bone matrix. The primitive osteogenic cells are present in enormous numbers in an irregular fashion, but do not form bony trabeculæ unless ascorbic acid is provided. In this we are in agreement with Wolbach and Howe as regards the observations, but not as regards their interpretation. Our observations on the teeth of guinea-pigs lead us to believe that the cross-sections shown in the article of Menkin, Wolbach and Menkin (1933) are misleading. Fig. 1 of that article is not typical of scurvy, and Fig. 2 is only typical of scurvy and shows no definite evidence of healing. As Ham and Elliott have pointed out, the separation of dentine from pulp is an artefact and has nothing to do with scurvy. Evidences of either scurvy or of the healing process are better seen in longitudinal section of molar teeth than in cross-sections of incisors. The early stage of healing is at the bottom of the tooth and, as one might expect, the abnormal dentine laid down during scurvy persists in the tops of the teeth for 3–4 weeks after administration of ascorbic acid is begun. The evidence of healing is a return of the odontoblasts to normal, with subsequent formation of normal dentine. Unless cross-sections are taken at low levels, this evidence would not be secured. The presence of calcific scar-tissue, as seen by Wolbach and Howe, and referred to by Menkin, Wolbach and Menkin, is not evidence of a jellation produced in healing, but is the result of the previous deficiency. This interpretation removes the chief evidence for the jellation theory.

In our opinion the explanation of Fish and Harris that scurvy results in some injury to odontoblasts and as a result an abnormal type of dentine is laid down best suits the observations. We would go further and, as a result of our studies, say that new bone-formation from osteoblasts is likewise of an abnormal type. This gives the *Gerustmark*, but lends no support to Wolbach and Howe's theory that this substance is a swollen tissue lacking a gel factor. In healing, the change is more apparent at the ordinary site of ossification, and only later does the *Gerustmark*, now left behind the growing layer, become

removed. In other words, when ascorbic acid is present the osteoblasts and primitive bone-marrow cells reach the layer of calcified cartilage and the osteoblasts exude their osteomucin material. When a deficiency exists these osteoblasts or mesenchymal cells accumulate as a ground-work, and do not form a normal bone matrix. Once ascorbic acid is given, the new osteoblasts at the growing area assume a normal function again and only later does the abnormal reaction (*Gerustmark*) disappear.

In our experiments an acute deficiency of ascorbic acid in guinea-pigs causes no characteristic histological change in tissues other than in bones and teeth.

SUMMARY.

Tissue changes resulting from an acute deficiency of ascorbic acid in guinea-pigs, using the paired feeding technique so that the variable factor of inanition may be controlled, show that the principal abnormalities are in the bones and teeth. In both these cases the vitamin deficiency causes a failure in those special cells which are concerned with the formation of calcific tissue. No evidence has been secured to support the jellation theory. Characteristic changes in other tissues have not been obtained. The inanition which is coincident with the development of ascorbic acid deficiency is not responsible for any of the observed histological changes.

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NEUTRALIZING AND COMPLEMENT-FIXING PROPERTIES OF ANTISERA PRODUCED BY FRACTIONATED EXTRACTS OF A NON-FILTERABLE DIBENZANTHRACENE FOWL SARCOMA.

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In previous experiments (Foulds, 1937) sera which neutralized filtrates of Rous sarcoma No. 1 were produced by injecting Berkefeld filtrates of the fowl dibenzanthracene sarcoma, RFD2, into rabbits; filtrates of RFD2 never produced tumours in fowls. These observations have been confirmed and extended by immunizing rabbits with extracts of RFD2 filtered through graded collodion membranes and by carrying out complement-fixation tests as well as neutralization tests with the sera.

PRODUCTION OF ANTISERA.

The method of preparing filtrates was based on Elford and Andrewes' (1935) determination of the particle size of the Rous agent by filtration through Elford's " Gradocol " membranes.

A 2·5 per cent. extract of fresh tumour in a mixture of equal parts of broth and 0·85 per cent. saline was passed through a sand and paper-pulp filter. A portion of the filtrate was injected into rabbit No. 1 and another portion was filtered through a gradocol membrane having an average pore diameter of about 0·8μ (0·79μ–0·82μ). A portion of the 0·8μ filtrate was injected into rabbit No. 2 and the remainder was filtered through a membrane having an average pore diameter of 0·14μ–0·15μ. The 0·14μ filtrate was injected into rabbit No. 3. The first series of injections comprised eight intravenous doses increasing from 1·0 c.c. to 5·5 c.c. The injections were made thrice weekly and the rabbits were bled seven days after the last injection; the details are recorded in Table I. A month later a series of four intraperitoneal injections increasing from 8 to 30 c.c. was given. For this series it was necessary to modify the method of preparing filtrates. Sand and paper-pulp filtrates clogged the 0·8μ membranes and increasing amounts of filtrates could not be obtained. The tumour-extracts, therefore, were filtered rapidly through a sand and paper-pulp filter and then filtered through a Berkefeld N candle. Berkefeld filtrate was injected into rabbit No. 1 and used for the preparation of the membrane filtrates. The same procedure was used in a third series of four intraperitoneal injections of 25–30 c.c. given after an intermission of five weeks. Seven weeks later a fourth series of three intraperitoneal injections of 24–35 c.c. was given.

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TABLE I.—*Immunization of Rabbits with Filtrates of RFD2 Sarcoma.*

Date.	Rabbit 1. Sand and paper-pulp filtrate c.c. intravenous.	Rabbit 2. 0·8 μ gradocol filtrate c.c. intravenous.	Rabbit 3. 0·14 gradocol filtrate c.c. intravenous.
14.i.39	1	1	1
17.i.39	2	2	2
19.i.39	2·5	2·5	2·5
21.i.39	3	3	3
23.i.39	3·5	3·5	3·5
25.i.39	4	4	4
27.i.39	5	5	5
30.i.39	5·5	5·5	5·5
6.ii.39	Bled	Bled	Bled
	Berkefeld filtrate c.c. intraperitoneal.	0·8μ gradocol filtrate c.c. intraperitoneal.	0·14 μ gradocol. filtrate c.c. intraperitoneal.
27.ii.39	8	8	8
1.iii.39	15	15	15
3.iii.39	23	23	23
6.iii.39	30	30	30
13.iii.39	Bled	Bled	Bled
12.iv.39	25	25	25
14.iv.39	30	30	30
17.iv.39	30	30	30
19.iv.39	30	30	30
26.iv.39	Bled	Bled	Bled
7.vi.39	24	24	24
9.vi.39	29	29	29
12.vi.39	35	35	35
19.vi.39	Bled	Bled	Bled

Filtrates of Rous sarcoma No. I were prepared in the same way and tested for infectivity in chickens. In each of four experiments the 0·8μ filtrate was highly active ; in three experiments the 0·14μ filtrate was inactive, but in one experiment a 0·14μ filtrate produced tumours though the activity compared with corresponding 0·8μ filtrate was greatly reduced. In Elford and Andrewes' experiments, filtrates through membranes having an average pore diameter of less than 0·25μ were not active.

NEUTRALIZATION OF FILTRATES OF ROUS SARCOMA I.

Neutralization tests were carried out as described previously (Foulds, 1937). After the first series of injections none of the sera reduced the activity of a Rous filtrate. After the second series, the serum of Rabbit 1 partially neutralized a Rous filtrate and the sera of Rabbits 2 and 3 had no effect. A highly active Rous filtrate was neutralized almost completely by serum taken from Rabbit 1 after the third series of injections, and its activity was reduced

to a much less extent by the sera of Rabbits 2 and 3. All the sera now fixed complement when added to extracts of normal fowl tissues (Table V). After the fourth series of injections, the serum of Rabbit 1 completely neutralized a Rous filtrate of low potency and the sera of Rabbits 2 and 3 had a slight effect ; the sera used in this experiment were previously absorbed with minced chick embryo but complement-fixation tests showed that the removal of " fowl " antibodies was not complete. The experiment was repeated with a Rous filtrate of higher potency and sera which had been absorbed with extract of normal chicken liver ; the serum of Rabbit 1 greatly reduced the activity of the Rous filtrate, but the sera of Rabbits 2 and 3 had no apparent effect. It is concluded, therefore, that the strong neutralizing properties of Rabbit 1 serum were due to specific tumour antibodies which were not " anti-fowl ". The weak neutralizing properties of the sera of Rabbits 2 and 3 might be attributed to similar antibodies present in so small amount that they were absorbed non-specifically by normal fowl tissues, but it is more probable that they were due to " fowl " antibodies.

COMPLEMENT-FIXATION REACTIONS.

The sera were used in complement-fixation tests with antigens of the same kind as those injected into rabbits and with similar antigens prepared from Rous sarcoma No. 1. The tests were carried out by the methods described by Dmochowski (1938) and Dmochowski and Knox (1939).

After the first series of injections, the sera of Rabbits 1 and 2 reacted with 10 per cent. Berkefeld filtrates of Rous sarcoma I ; the serum of Rabbit 3 reacted slightly with one filtrate but not at all with the two others which were tested (Table II). Similar results were obtained with 10 per cent. Berkefeld filtrates of RFD2 sarcoma. The sera were too weak to yield satisfactory results with 2·5 per cent. filtrates of RFD2 unless the filtrates were concentrated. A 0·8μ gradocol filtrate, which had been concentrated by reducing its volume by filtration through a 0·14μ membrane, reacted with the sera in the same way as 10 per cent. Berkefeld filtrate (Table II). All subsequent tests were carried out with 2·5 per cent. filtrates.

After the second course of immunizing injections, the sera of Rabbits 1 and 2 reacted strongly with Berkefeld and 0·8μ gradocol filtrates of Rous sarcoma, but either weakly or not at all with 0·14μ filtrates, whereas the serum of Rabbit 3, previously inert, now reacted strongly with each of the three filtrates (Table III). Similar results were obtained when RFD2 filtrates were used as antigens, the 0·14μ filtrates reacting with the serum of rabbit 3 only (Table IV). The 0·14μ gradocol filtrates thus contained an antigen which reacted with antiserum elicited by similar filtrates but not with antisera elicited by Berkefeld or 0·8μ gradocol filtrates although, owing to the method of preparation, all constituents of 0·14μ filtrates were necessarily present in equal or greater concentration in the Berkefeld and 0·8μ gradocol filtrates. It was inferred that 0·14μ filtrates contained an antigen whose capacity for stimulating antibody-formation was given full scope in Rabbit 3, but suppressed in Rabbits 1 and 2 by the simultaneous action of another and stronger antigen which had already evoked antibodies at the first bleeding. The suppression

TABLE II.—*Complement-fixation Tests with Sera Obtained after First Series of Immunizing Injections.*

	Rabbit 1. Serum dilutions.			Rabbit 2. Serum dilutions.			Rabbit 3. Serum dilutions.		
	1/8.	1/16.	1/32.	1/8.	1/16.	1/32.	1/8.	1/16.	1/32.
Rous sarcoma :									
10 per cent. Berkefeld filtrate	+++	+++	+++	+++	+++	−	−	−	−
RFD2 sarcoma :									
10 per cent. Berkefeld filtrate	+++	+++	+++	+++	+++	−	−	−	−
2·5 ,, ,, ,,	+	−	−	−	−	−	−	−	−
0·8μ Gradocol filtrate	−	−	−	−	−	−	−	−	−
Concentrated 0·8μ Gradocol filtrate	+++	++	±	+++	±	−	−	−	−
0·14μ Gradocol filtrate	−	−	−	−	−	−	−	−	−

TABLE III.—*Complement-fixation Tests with Rous Sarcoma Filtrates and Sera Obtained after the Second Series of Immunizing Injections. In other experiments the sera of Rabbits 1 and 2 did not react with 0·14μ filtrates of Rous sarcoma. The table also shows tests made with extract of normal fowl liver and the same sera, and tests of normal tissue and tumour extracts with an "anti-fowl" serum prepared by injecting washed red blood-cells of fowls into a rabbit.*

	Rabbit 1. Serum dilutions.			Rabbit 2. Serum dilutions.			Rabbit 3. Serum dilutions.			"Anti-fowl" serum dilutions.		
	1/8.	1/16.	1/32.	1/8.	1/16.	1/32.	1/8.	1/16.	1/32.	1/8.	1/16.	1/32.
Rous sarcoma :												
Berkefeld filtrate	+++	++	−	+++	+++	−	+++	+++	−	+	±	−
0·8μ Gradocol filtrate	++	±	−	+++	++	−	+++	+++	−	−	−	−
0·14μ ,, ,,	±	−	−	++	−	−	+++	+++	−	−	−	−
Extract of normal fowl liver	−	−	−	−	−	−	−	−	−	+++	+++	+++

TABLE IV.—*Complement-fixation Tests with RFD2 Filtrates and Sera Obtained After the Second Series of Immunizing Injections.*

	Rabbit 1. Serum dilutions.			Rabbit 2. Serum dilutions.			Rabbit 3. Serum dilutions.		
	1/8.	1/16.	1/32.	1/8.	1/16.	1/32.	1/8.	1/16.	1/32.
RFD2 sarcoma :									
Berkefeld filtrate	+++	+++	++	+++	+++	++	+++	+++	++
0·8μ Gradocol filtrate	+++	+++	++	+++	++	++	+++	+++	++
0·14μ ,, ,,	−	−	−	−	−	−	+++	+++	±

of the antigenic power of the weaker of two antigens used simultaneously for immunization is well known.

After the third series of injections each serum reacted, in about the same degree, with extracts of normal fowl tissue whereas previously none of the sera did so. The sera were absorbed with extract of normal fowl liver and the reactions with normal fowl liver were thereby abolished without altering the reactions with tumour filtrates (Tables V and VI). The reactions were similar to those observed at the previous bleeding except that 0·14μ gradocol filtrates of RFD2 now reacted equally and strongly with each serum, whereas 0·14μ filtrates of Rous sarcoma reacted, as before, more strongly with the serum of Rabbit 3 than with the sera of Rabbits 1 and 2. This difference between 0·14μ filtrates of RFD2 and Rous sarcoma was not observed in tests with absorbed sera obtained after the fourth series of injections. These sera yielded no new information. It seemed that the injections had not increased the antibodies or that the sera had deteriorated during the several weeks of storage which elapsed before the tests could be carried out.

DISCUSSION.

The experiments confirm the previous demonstration that non-infective extracts of the dibenzanthracene sarcoma RFD2 elicit antisera which inactivate filtrates of Rous sarcoma No. 1. The specific neutralizing antibodies which were not removed by absorption with normal fowl tissues were present in Rabbit 1, which received sand and paper-pulp and Berkefeld filtrates of RFD2, but not in Rabbit 2, which received 0·8μ gradocol filtrates. Similar 0·8μ filtrates of Rous sarcoma were always infective and antigenic particles in RFD2, comparable in size with the Rous agent, would be expected to enter 0·8μ filtrates and evoke antibodies in Rabbit 2. It is possible that some particles of that size were held back, for consistent filtrations depend on various conditions of material and procedure which, probably, were not always favourable, especially in the first stage of immunization, but it seems improbable that the total dose of antigen received by Rabbit 2 was sufficiently reduced in this way to account for the absence of neutralizing antibodies. The possibility that Rabbits 1 and 2 reacted unequally to similar stimulation needs consideration ; it is noteworthy, however, that their sera had similar complement-fixing properties. The provisional conclusion is that the antigenic particles of RFD2 which elicited neutralizing antibodies were larger than the infective agent of Rous sarcoma No. 1.

Correlation between neutralization and complement-fixation tests was not established. The complement-fixation tests indicated that Rous sarcoma No. 1 and RFD2 sarcoma had two common antigens which, by the methods used, were not demonstrated in normal fowl tissues. One antigen passed through 0·8μ membranes but not through 0·14μ membranes and, therefore, was comparable in size with the Rous agent ; the other passed through 0·14μ membranes and was probably smaller than the agent. Under the conditions of the experiments Rous agent penetrated 0·14μ membranes infrequently and to a slight extent, but we are not satisfied that an equal escape of particles into

TABLE V.—*Complement-fixation Tests with Rous Sarcoma Filtrates and Sera Obtained after the Third Series of Immunizing Injections. Each serum now reacts with normal fowl tissue extract.*

	Rabbit 1. Serum dilutions.			Rabbit 2. Serum dilutions.			Rabbit 3. Serum dilutions.		
	1/10.	1/20.	1/40.	1/10.	1/20.	1/40.	1/10.	1/20.	1/40.
Rous sarcoma:									
Berkefeld filtrate	+++	+++	+++	+++	+++	++	+++	+++	+
0·8μ Gradocol filtrate	+++	+++	+++	+++	+++	++	+++	+++	+ +
0·14μ ,, ,,	++	±	−	++	±	−	+++	+++	+++
Extract of normal fowl liver	+++	+++	++	+++	++	±	+++	+++	±

TABLE VI.—*Complement-fixation Tests Carried out at the same time as those Shown in Table V. The same antigens were used but the sera had been absorbed with a dried extract of normal fowl liver. The sera no longer react with normal fowl tissue extract, but the reactions with tumour filtrates are unchanged.*

	Rabbit 1. Serum dilutions.			Rabbit 2. Serum dilutions.			Rabbit 3. Serum dilutions.		
	1/10.	1/20.	1/40.	1/10	1/20.	1/40.	1/10.	1/20.	1/40.
Rous sarcoma:									
Berkefeld filtrate	+++	+++	+++	+++	+++	+++	+++	+++	++
0·8μ Gradocol filtrate	+++	+++	+++	+++	+++	++	+++	+++	++
0·14μ ,, ,,	++	+	±	++	+	−	+++	+++	+
Extract of normal fowl liver	−	−	−	−	−	−	−	−	−

0·14μ filtrates of RFD2 would be sufficient to account for the production of antibodies.

The neutralization and complement-fixation tests together provide evidence that RFD2 sarcoma contains antigenic particles which are similar in size to the infective agent of Rous sarcoma and other antigenic particles, some of which are larger than the agent and some smaller. It is possible that the smaller antigenic particles result from disintegration of the larger. It was suggested previously (Foulds, 1937) that the filterability of fowl tumours depends on the dissociation of a virus-protoplasm complex in a particular way. The new observations are consistent with the hypothesis, but do not establish it. The inconclusive results are recorded to illustrate the complexity of the serological reactions of fowl tumours ; more accurate methods than we have used are needed to analyse it.

SUMMARY.

The serum of a rabbit, which received injections of sand and paper-pulp and Berkefeld filtrates of the non-filterable dibenzanthracene fowl sarcoma RFD2, neutralized filtrates of Rous sarcoma No. 1 ; the neutralizing power was not abolished by absorption with normal fowl tissues. The sera of rabbits which received filtrates of the same tumour prepared by means of collodion membranes having average pore diameters of 0·8μ or 0·14μ respectively had weak neutralizing properties which were abolished by absorption with normal fowl tissues. The antigen which elicited neutralizing antibodies, other than "fowl" antibodies, was apparently larger than the infective agent of Rous sarcoma.

Complement-fixation tests indicated that RFD2 and Rous sarcoma contained two antigens which were not demonstrated in normal fowl tissues ; one antigen was comparable in size with the infective agent of Rous sarcoma and the other was smaller.

The relationship between the neutralizing and the complement-fixing antibodies was not established.

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A COMPLEMENT-FIXING ANTIGEN COMMON TO FILTERABLE AND NON-FILTERABLE TUMOURS OF FOWLS.

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CELL-FREE filtrates of the filterable tumours of birds contain, in addition to easily identifiable species-specific elements, at least one other antigenic factor (Gye and Purdy, 1931). The balance of evidence favours the view that this factor is not species-specific, and it may be conveniently referred to as "extrinsic" to indicate at least that it is not detectable in the normal tissues of the host, and is detectable in filtrates of duck-grown, as well as of fowl-grown tumours. Subsequent experiments by Andrewes (1936), Gye and Foulds (1936) and Foulds (1937) indicated that filtrates of certain non-filterable sarcomas of fowls induced by chemical compounds share with filtrates of the filterable tumours a common antigenic factor which is the same as, or at least closely related to, the extrinsic factor described by Gye and Purdy. The evidence for these conclusions rests on the results of neutralization experiments with sera obtained either from tumour-bearing birds, or from animals of a different species immunized with normal fowl antigens on the one hand and with tumour filtrates on the other. This paper describes preliminary experiments in which by means of a complement-fixation reaction it has been possible to confirm conclusions which have hitherto rested solely on evidence provided by neutralization experiments.

EXPERIMENTAL.

The serum used came from a rabbit immunized with filtrates of the non-filterable dibenzanthracene sarcoma RFD2 passed through collodion membranes of average pore diameter 0·8μ. It is referred to as anti-0·8μ RFD2 serum. It had been heated at 55°–56° C. for half an hour. The rabbit received several courses of injections; in most experiments serum obtained after the first course was used, but serum obtained from the same rabbit after a second and a third course was also used. As will be seen from the discussion, it differed from the serum obtained after the first course. The details of the immunization of the rabbit are given by Foulds and Dmochowski (1939).

The antigens were usually filtrates of tumours or of organs prepared in a strength of 10 per cent. in saline and filtered through paper pulp and then through Berkefeld N candles. Tests of tumour-producing activity were made by injecting doses of 1 c.c. into the breasts or legs, or both, of birds from 6–10 weeks old.

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The technique of complement-fixation was the same as that described by Dmochowski (1938). *No difficulty arose from anti-complementary properties of the antigens,* as a careful preliminary antigen titration determined the quantity of antigen which could safely be used. The volumes used in the test were: Serum 0·5 c.c., antigen 0·1–0·3 c.c., complement 0·25 c.c., sensitized sheep red corpuscles 0·5 c.c. The total volume was brought to 1·5 c.c. with saline if necessary; when 0·3 c.c. of antigen was used the total volume was 1·55 c.c. Antigen and serum controls were included in every experiment. After preliminary titration of complement, 5 M.H.D. were used in the experiments. Falling dilutions of serum were titrated against a constant volume of antigen. The sheep red blood-cells in a strength of 3 per cent. were sensitized with 5 M.H.D. of rabbit hæmolysin of high titre prepared in the laboratory. Saline, serum, antigen and complement were added in that order. Fixation was allowed to occur for 50 minutes to 1 hour at 37° C.; the sensitized sheep corpuscles were then added and readings were taken after 15 minutes at 37° C. Workers with vaccinia and other viruses have found that long fixation in the cold room gives much higher titres than does fixation for a short time at room temperature or at 37° C., but the influence of variations in time and temperature on the particular system used in these experiments has not been tested, as the conditions chosen gave satisfactory results.

RESULTS.

Complement-fixation with Filtrates of Filterable Tumours.

Rous sarcoma No. 1.

Table I gives the results of ten consecutive experiments in which Berkefeld filtrates of different Rous tumours were tested. It shows a rough correlation between the activity of filtrates and the presence and strength of the reaction

TABLE I.—*Activity of Different Rous Filtrates and Complement-fixation with Anti-0·8μ RFD2 Serum.*

No. of tumour.	Activity.	Complement-fixation serum dilutions.		
		1/8.	1/16.	1/32.
6440	++	+++	+++	±
6506	+++	++	+	—
6604	++	+++	++	—
6439	+++	++	+	—
6372	++	—	—	—
6539	+	—	—	—
6423	(±)	—	—	—
6603	—	—	—	—
6352	—	—	—	—
6356	—	—	—	—

The relative activity of the different filtrates is indicated by + signs. Degrees of complement-fixation are indicated as follows:

+++ = complete fixation.
++, +, ± = partial fixation.
— = no fixation (complete hæmolysis).

with anti-0·8μ RFD2 serum. Table II gives the results of a few experiments in which some of the filtrates which appear in Table I were tested with a high titre anti-fowl serum as well as with anti-0·8μ RFD2 serum. The importance of this comparison will be discussed later. Table II also shows that filtrates of normal fowl organs used as controls give a negative reaction with anti-0·8μ RFD2 serum, but positive with anti-fowl serum.

Mill Hill 2 and Fujinami tumours.

Filtrates of Mill Hill 2 and Fujinami tumours were also tested with anti-0·8μ RFD2 serum, and with these, too, it was found that the strength of the complement-fixation varied with the activity of the filtrate.

Duck-grown Fujinami tumour.

The filtrates in the above-mentioned experiments were all from fowl-grown tumours ; but in one experiment filtrates of a duck-grown as well as of a fowl-grown Fujinami tumour were tested for complement-fixation with anti-0·8μ RFD2 serum and with anti-fowl serum. The duck tumour used had been kindly supplied by Dr. Purdy, who had passaged it through several generations of ducks. The antigens used were paper pulp, Berkefeld and 0·8μ membrane filtrates of each tumour, and the result is shown in full in Table III. It will be noted that the paper pulp filtrate of the duck tumour gives complete fixation with anti-fowl serum in a dilution of 1/16. The paper-pulp filtrate of the fowl-grown tumour gives positive fixation with anti-fowl serum in a dilution of 1/64. It is unfortunate that the reaction in this particular experiment was not titrated to its end-point ; but in other experiments paper-pulp filtrates have been found to give complement-fixation with the same anti-fowl serum in dilutions as high as 1/640–1/720, and it can be safely assumed that the paper-pulp filtrate of the fowl-grown tumour would have given complement-fixation with anti-fowl serum in a dilution much higher than 1/64. It is evident, however, that when antigens containing large amounts of protein are used there is a certain amount of cross-reaction between duck protein and anti-fowl serum. The Berkefeld and 0·8μ membrane filtrates of the two tumours, however, give clean results. All are positive with anti-0·8μ RFD2 serum, but only the filtrates of the fowl-grown tumour react with anti-fowl serum.

Complement-fixation with Filtrates of RFD2 and with Heat-inactivated Rous Filtrates.

Table IV shows that a positive complement-fixation reaction is given with anti-0·8μ RFD2 serum by some, though not by all, Berkefeld filtrates of RFD2, the tumour from which filtrates were prepared for immunization. The reaction is also positive when Rous filtrates, which give a positive reaction in the fresh state, are tested after inactivation by heating to 60° C., or even higher.

TABLE II.—*Complement-fixation of Different Rous Filtrates with Anti-0·8μ RFD2 Serum and with Anti-fowl Serum.*

Antigens.	Anti-0·8μ RFD2 serum. 1/4.	1/8.	1/16.	1/32.	Anti-fowl serum.. 1/4.	1/8.	1/16.	1/32.	1/64.	
6352	−	−	−	−	±	±	−	−	..	
6356	−	−	−	−	±	±	−	−	..	
6423	..	−	−	−	..	++	−	−	..	
6440	+++	+++	+++	+	+++	+++	+++	+++	..	
6604	..	+++	+	−	..	+++	+++	+++		Positive to 1/400.
9	..	+++	−	−	..	−	−	−		Part of one
Normal liver	..	−	−	−	..	+++	+++	+++		experiment.
3186	..	+++	+++	−	..	+	±	−		Ditto.
Normal liver	..	−	−	−	..	+++	+++	+++		

TABLE III.—*Complement-fixation by Filtrates of Duck and Fowl Fujinami Tumour with Anti-0·8μ RFD2 and with Anti-fowl Serum.*

Antigens.	Anti-0·8μ RFD2 serum. 1/8.	1/16.	1/32.	1/64.	Anti-fowl serum. 1/8.	1/16.	1/32.	1/64.	Antigen controls. With complement.	Without complement.
Fowl-grown Fujinami: Paper-pulp filtrate	+++	+++	+++	++	+++	+++	+++	+++	−	+++
Fowl-grown Fujinami: Berkefeld ,,	+++	+++	++	−	+++	+++	+++	+	−	+++
Fowl-grown Fujinami: 0·8μ membrane filtrate	+++	+++	+++	−	+++	+++	+++	−	−	+++
Duck-grown Fujinami: Paper-pulp filtrate	+++	+	−	−	+++	+++	++	−	−	+++
Duck-grown Fujinami: Berkefeld ,,	+++	++	−	−	−	−	−	−	−	+++
Duck-grown Fujinami: 0·8μ membrane filtrate	+++	++	−	−	−	−	−	−	−	+++
Serum controls (with complement)	−	−	−	−	−	−	−	−	−	+++

TABLE V.—*Absorption of Anti-0·8μ RFD2 Serum with Different Antigens.*

Antigens.	Unabsorbed serum. 1/8.	1/16.	1/32.	Absorbed with— Rous filtrate. 1/8.	1/16.	1/32.	Embryo. 1/8.	1/16.	1/32.	Liver. 1/8.	1/16.	1/32.
Rous 6440	+++	+++	+	+++	−	−	+++	+++	+	+++	+++	+
,, heated	+++	+++	±	+++	−	−	+++	+++	±	+++	+++	±

TABLE IV.—*Complement-fixation of RFD2 and Heat-inactivated Rous Filtrates with Anti-0·8μ RFD2 Serum.*

	Antigens.	Serum dilutions. 1/8.	1/16.	1/32.
RFD2	6425	+++	+++	±
	6518	−	−	−
	6519	−	−	−
	6426	−	−	−
	3203	+++	+++	−
	3199	+++	+++	−
	3197	+	−	−
	3164	+++	−	−
Rous	6440 56° C. ½ hour	+++	+++	+
	„ 85° C. ½ hour	+++	+++	+
	„ unheated	+++	+++	+

Absorption of Anti-0·8μ RFD2 Serum with Normal Fowl Tissues and with Positive Rous Filtrates.

Table V shows that when anti-0·8μ RFD2 serum is absorbed with Berkefeld filtrates of chick embryo and of normal fowl liver, no reduction occurs in the titre of the complement-fixation reaction with a Rous filtrate which gives a positive reaction with the unabsorbed serum. However, when the serum is absorbed with the same Rous filtrate a definite reduction in the titre of the reaction occurs.

DISCUSSION.

The complement-fixation reaction here described appears to have a certain practical value. By its use the activity of different filtrates not only of the Rous tumour, but also of Mill Hill 2 and Fujinami tumours, can be roughly predicted before the results of biological tests are available. It is important to note that this only appears to hold when anti-0·8μ RFD2 serum obtained after the first series of injections is used. About thirty complement-fixation tests were done with this particular sample of serum. No more could be done because the supply of serum was limited, and it was discovered that when serum obtained after the second series of injections was used, the complement-fixation reaction with anti-0·8μ RFD2 serum was less reliable as an indicator of activity than when serum of the first bleeding was used. When serum obtained after the third series of injections was used it was found to give a reaction with inactive as well as with active filtrates of the Rous tumour ; but since the serum at that stage gave a strong reaction with filtrates of normal fowl tissues it is evident that it had acquired anti-fowl properties, and therefore could react with species-specific antigens in tumour filtrates.

It is, however, reasonably certain that the serum of the first bleeding does

detect an antigen which is not species-specific. There are three pieces of evidence which point to this :

(1) Filtrates of normal fowl organs give a negative complement-fixation reaction with anti-0·8μ RFD2 serum, but a strong positive reaction with anti-fowl serum. Tumour filtrates, on the other hand, give a positive reaction with anti-0·8μ RFD2 serum, while in some experiments the same filtrates give a negative reaction with an anti-fowl serum with which filtrates of normal organs give a strong positive reaction.

(2) Absorption with normal fowl tissues fails to influence the reaction with anti-0·8μ RFD2 serum (Table V).

(3) Filtrates of duck-grown as well as of fowl-grown Fujinami tumour give a positive reaction with anti-0·8μ RFD2 serum.

The first two pieces of evidence support the view that the complement-fixing antigen is not species-specific in the sense that it is not derived from normal elements of the host. It might, however, still be an abnormal derivative of fowl cells. The positive reaction with filtrates of duck-grown Fujinami tumour makes this unlikely, and suggests that the antigen revealed by complement-fixation is extrinsic in origin.

Since the serum used in these experiments was directed against 0·8μ filtrates of RFD2 tumours, the positive reaction with RFD2 filtrates was to be expected. The reaction varies in intensity with different RFD2 filtrates and with some is negative ; it would appear that the complement-fixing antigen may be present in greater or less amount in different RFD2 filtrates or may even be absent. It is difficult to explain the meaning of this at present. On the other hand, the reaction between anti-0·8μ RFD2 serum and active filtrates of the filterable tumours seems to indicate that the complement-fixing antigen is at least related to the active tumour-producing agent. It cannot, however, be the active agent itself, since it is also present in filtrates of RFD2, a tumour which has never been successfully filtered, and in filtrates of filterable tumours inactivated by heat.

It is clear then that the complement-fixation reaction reveals an antigenic factor common to filtrates of filterable and of non-filterable tumours. It does not follow that this antigenic factor is identical with the common factor deduced by Gye, Andrewes and Foulds from the results of neutralization experiments. Neither factor appears to be species-specific, while the evidence provided by the experiment with filtrates of duck-grown Fujinami tumour suggests that the complement-fixing antigen is extrinsic. In this respect it closely resembles the extrinsic antigen described by Gye and Purdy in filtrates of filterable tumours. It is not claimed that the complement-fixing antigen is entirely free from species-specific elements, nor that the anti-0·8μ RFD2 serum is entirely free from anti-fowl elements. An anti-fowl serum of high titre shows that 0·8μ filtrates of RFD2 may contain considerable amounts of fowl protein, and even though after the first course of injections the anti-0·8μ RFD2 serum contained no detectable anti-fowl antibodies, it certainly developed them to a considerable titre after the third course, and cannot be assumed to have been entirely free from them even in earlier stages. A serum containing antibodies to an extrinsic factor and entirely free from species-specific antibodies could only be obtained if it were possible to prepare the extrinsic

factor in a pure form free from all species-specific elements and fully antigenic.

Since only crude tumour filtrates have been used in these experiments, it is impossible to say whether the extrinsic element would be completely antigenic if it were obtained in a state of purity. It is hoped by preparing sera against filtrates of the filterable tumours and also against material freed as far as possible from fowl protein, to learn more about the extrinsic element in tumour filtrates and about the immunological relation between filterable and non-filterable tumours.

SUMMARY.

Serum prepared by immunizing a rabbit with 0·8μ membrane filtrates of the non-filterable tumour RFD2 gives complement-fixation with active filtrates of Rous and Mill Hill 2 tumours and of fowl- and duck-grown Fujinami tumours.

The reaction has a practical value in making it possible to predict the relative activity of different filtrates before the results of activity tests are available.

Since RFD2 filtrates and heat-inactivated Rous filtrates also give a positive complement-fixation reaction, it is evident that both filterable and non-filterable tumours share a common antigenic factor.

Reasons are given for concluding that the antigenic factor detected by complement-fixation is not predominantly species-specific. It is briefly described as a complement-fixing antigen.

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OBSERVATIONS ON A REACTION BETWEEN THE LETHAL TOXIN OF *CL. WELCHII* (TYPE A) AND HUMAN SERUM.

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In studying the growth of *Cl. welchii* (Type A) in human serum, a change in the appearance of the serum was observed and investigated.

If human serum is inoculated with *Cl. welchii* and incubated for 16 hours in an anaërobic jar, the medium is seen to have become opalescent with the uppermost layer more opaque than the rest of the medium. If this serum culture is centrifuged, three zones are clearly seen—the bottom layer consists of bacilli, the middle layer of opalescent serum, and the top layer of a fat-like material.

It will be shown in this paper that—

1. This change in human serum is caused by the lethal toxin of *Cl. welchii* (Type A).
2. The reaction is inhibited specifically by the corresponding antitoxin.
3. Both toxin and antitoxin can be titrated by means of this reaction.

No previous description of this phenomenon has been found in the literature.

MATERIALS.

1. *Human serum.*—The serum should be free from fat, and therefore it is desirable to take the blood from an individual who has fasted for several hours. The serum is inactivated at 56° C. for one hour before using.

2. *Cultures.*—*Cl. welchii* Type A from (i) National Collection Type Cultures Nos. 2836, 2544, 2910, 274. (ii) 90 strains freshly isolated from the cervix uteri by Dr. Chesterman at The Women's Hospital, Crown St., Sydney. *Cl. welchii* Type B, Type C and Type D, sent by Dr. A. T. Wilsdon, Cambridge, to the McMaster Laboratory, Sydney.

Other species of the genus clostridum were also used, mainly from N.C.T.C.

3. *Toxins.*—Fluid toxins were prepared by cultivating various strains of *Cl. welchii* in Pope's broth with fresh sterile liver or meat. Dried toxins of *Cl. welchii* (Type A) were obtained from the Commonwealth Serum Laboratories, Melbourne, and from the Institut für experimentelle Therapie, Frankfurt am Main. These dried toxins had already been titrated by mouse test in the above laboratories, and their toxicity expressed in m.l.d. per mg.

4. *Antitoxic sera.*—*Cl. welchii* (Type A) antitoxic sera were obtained from

the Commonwealth Serum Laboratories, Melbourne; from the Wellcome Physiological Research Laboratories, Beckenham, Kent; and from the Institut für experimentelle Therapie, Frankfurt am Main. These antitoxic sera had been titrated by mouse test in the above laboratories, and their strength expressed in international units per c.c. The *Cl. welchii* types B, C and D antitoxic sera were obtained from Dr. A. T. Wilsdon, Cambridge. These sera were prepared in sheep.

TECHNIQUE.

Cultural reaction in human serum.—0·3 c.c. of a mixture of equal parts of inactivated human serum and nutrient broth is pipetted into a test-tube, approximately 5 mm. internal diameter. 0·3 c.c. of the same mixture is pipetted into a similar test-tube, to the contents of which is added one drop of *Cl. welchii* (Type A) antitoxic serum containing at least 20 units per c.c. Both tubes are inoculated with one drop of culture of *Cl. welchii* and incubated for 16 hours in an anaërobic jar. While the great majority of strains of *Cl. welchii* grow in pure serum, a small percentage will grow only in the serum-broth mixture. The latter strains do not readily produce a stormy clot in milk, although by all other criteria, biological and serological, they are true *Cl. welchii*.

After incubation the tubes are examined. In the tube without antitoxin there is an obvious opalescence with a fatty layer at the top of the medium. The bacteria are seen at the bottom of the tube, although in some mucoid strains they are suspended in the medium. If doubt exists whether an opalescence in the medium is due to the suspended bacteria or to a change in the medium, centrifugation will clear up the doubt. After centrifugation there will be found a fatty layer at the top of the medium, and the bacteria will be seen at the bottom of the tube. In the tube with antitoxin, the medium remains clear with a sediment of bacteria. The cultural reaction is shown in Fig. 1.

Titration of toxin in vitro.—The general principle of the technique is to add falling quantities of toxin to a constant amount of human serum, and after 16 hours' incubation to note the least amount of toxin which causes a visible change in this amount of human serum. A toxin whose m.l.d. is very accurately known is taken as the standard toxin, and this standard toxin is always titrated simultaneously with any unknown toxin against the same human serum. The end-point of the reaction of the standard toxin is compared with the end-point of the unknown toxin, and the m.l.d. of the unknown toxin can then be calculated. The dried standard toxin is dissolved in saline immediately before use so that 1·0 c.c. of the solution contains 2·5 m.l.d. 0·2 c.c. amounts of human serum are added to a number of the small test-tubes described above, and the dissolved toxin is added in falling quantities starting with 0·1 c.c., then 0·09, 0·08, 0·072, 0·064, 0·056, 0·05, 0·044, 0·04, 0·036 c.c. Saline is added to the tubes, so that each tube contains a volume of 0·3 c.c. These quantities are small and perhaps increase the difficulty of accurate measurement. They were chosen to economize toxins and human serum. The contents of the tubes are mixed, and the tubes are incubated aërobically at 37° C. for

16 hours. They are then examined for any change in opacity. Degrees of opacity are read as +++, ++, +, ±, 0, +++ representing the maximum opacity in this test.

In the case of an unknown toxin, a preliminary titration can be carried out with 50 per cent. decreases, or a longer series with approximately 10 per cent. decreases, in parallel with the standard toxin. The m.l.d. of the unknown toxin is calculated by comparing the amounts of toxin in the end-point tubes of the unknown toxin series and the standard toxin series.

The in vitro *titration of* Cl. welchii *(Type A) antitoxin.*—The general principle of the technique is a preliminary short incubation of falling quantities of antitoxin with a constant quantity of toxin, after which a constant quantity of human serum is added to each tube. The tubes are incubated aërobically for 16 hours and read as in the toxin titration. The standard antitoxin is always titrated simultaneously with an unknown antitoxin, using the same

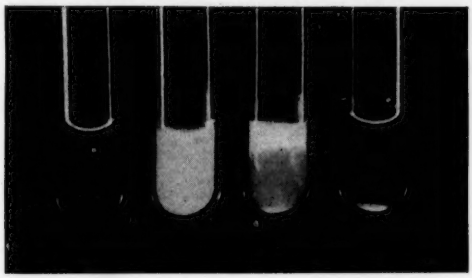

FIG. 1.—Cultural reaction of *Cl. welchii* in human serum.

1. Tube containing human serum-broth mixture immediately after inoculation with *Cl. welchii*. Medium is clear.
2. Tube containing human serum-broth mixture after inoculation with *Cl. welchii* and 16 hours' incubation in the anaërobic jar. Medium is now opalescent.
3. Tube containing human serum-broth mixture after inoculation with *Cl. welchii*, 16 hours' incubation in an anaërobic jar and centrifugation. The fatty layer is seen at the top of the tube, and the organisms at the bottom.
4. Tube containing human serum-broth mixture plus *Cl. welchii* antitoxin, after inoculation with *Cl. welchii* and incubation for 16 hours in an anaërobic jar. Medium is clear and the bacilli are seen at the bottom of the tube.

toxin. The amount of standard antitoxin which is just sufficient to inhibit the action of the toxin in causing opacity in the human serum is compared with the amount of the unknown antitoxin which has a similar inhibiting effect, and the strength of the unknown antitoxin can thus be calculated.

The dried toxin is dissolved in saline just before use, so that 1·0 c.c. of the toxin contains 20 m.l.d. 0·1 c.c. quantities of this toxin are then added to a series of tubes, which then receive falling quantities, 0·06, 0·055, 0·05, 0·045, 0·04, 0·036 c.c. of standard antitoxin diluted so that 1·0 c.c. contains 1 international unit. The volume of antitoxin in each tube is made up to 0·1 c.c. with saline solution. The tubes are incubated aërobically at 37° C. for $\frac{1}{2}$ hour, after which 0·1 c.c. of human serum is added to each tube. The

contents of the tubes are well mixed and the tubes are returned to the 37° C. incubator for 16 hours. The test is read as in the toxin titration. Further incubation does not change the reaction. The last tube in the series which shows no opacity is taken as the end-point of the reaction. With an unknown antitoxin a preliminary titration can be carried out with 50 per cent. decreases between the amounts of antitoxin, or a longer series can be set up with approximately 10 per cent. decreases.

As with the toxin titration, the total volume in each tube is small (0·3 c.c.) and can of course be increased, if such small volumes are found unduly difficult to measure accurately.

A photograph of an antitoxin titration is shown in Fig. 2.

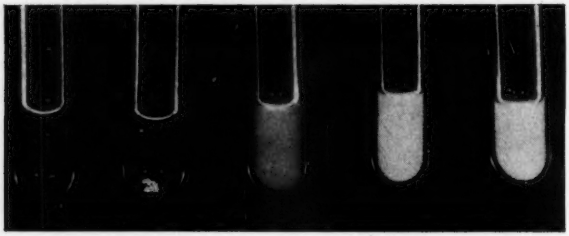

FIG. 2.—Titration of *Cl. welchii* Type A antitoxin in human serum.

Number of tube	1	2	3	4	5
Units of antitoxin 25	0·055	0·05	0·045	0·04	0·036
m.l.d. of toxin 187	2	2	2	2	2
Human serum in c.c.	0·1	0·1	0·1	0·1	0·1
Result	0	0	++	+++	+++

RESULTS.

A. *The Cultural Reaction in the Human Serum-broth Mixture.*

1. *The specificity of the reaction.*—All tested *Cl. welchii* cultures of known origin (Types A, B, C and D) produced the opacity in the medium, and the change was specifically inhibited by *Cl. welchii* antitoxin. The same result was obtained with the 90 strains isolated by Dr. Chestermann. These all showed the usual characters of *Cl. welchii*.

None of the following clostridia produced the opacity change in the medium : *Cl. œdematiens, septique, chauvœi, histolyticum, sporogenes, tertium, tetani, botulinum.*

The only organism found, other than *Cl. welchii*, which did change the medium in this way was a large motile Gram-positive aërobic spore-bearing rod, resembling the pseudo-anthrax bacilli. In this case the change in the medium was not inhibited by *Cl. welchii* antitoxin.

No antitoxin, other than *Cl. welchii* antitoxin, was able to inhibit the change in the medium produced by *Cl. welchii*.

Small amounts of *Cl. welchii* Types B, C and D antitoxin were available. Each of these antitoxins inhibited the change in the serum, whether it was caused by *Cl. welchii* Types A, B, C or D.

2. *The kind of serum which is affected by* Cl. welchii.—The following mammalian sera have been tested—man, monkey, dog, rabbit, guinea-pig, sheep, horse and ox. Human serum was the only one which was affected, with the exception that a specimen of monkey serum was slightly changed by *Cl. welchii* Type D, and the change inhibited by antitoxin. A few experiments indicated that chicken serum was slightly affected by *Cl. welchii* and the change inhibited by antitoxin.

55 different specimens of normal human serum were tested and all showed susceptibility to *Cl. welchii*. 78 different specimens of serum either from patients suffering from gas gangrene, or from symptomless patients carrying *Cl. welchii* in the cervix uteri, were tested. The only specimens which did not show the change were sera from patients who had been passively immunized with *Cl. welchii* antitoxin.

Serum was obtained from a number of untreated symptomless patients, three to four weeks after *Cl. welchii* had been isolated from the cervix uteri. All these specimens were affected by the cultural test, and even by the much more delicate toxin titration, the end-point of the toxin reaction in these sera being the same as in normal human sera. There was thus no evidence that antitoxin had been actively produced in these patients.

No opportunity presented itself of testing the serum from a convalescent untreated patient suffering from gas gangrene, as all these patients received large amounts of *Cl. welchii* antitoxin.

B. *Toxin Titration.*

Four different specimens of dried *Cl. welchii* (Type A) toxin were available from Frankfurt :

Toxin No.	M.L.D. (mg.)	M.H.D. (mg.)
187	0·15	5·0
377	0·35	0·05
484	0·2	5·0
590	1·0	0·05

Each of these toxins was dissolved in saline immediately before use, so that 1·0 c.c. of the solution of each toxin contained 2·5 m.l.d. The four solutions were then titrated for their action on human serum, and the results are shown in Table I. It will be seen that the end-points of the equivalent solutions of toxins 187, 377 and 484 are approximately the same, *i. e.* 0·18 m.l.d. is the least amount of toxin which will produce a visible reaction in 0·2 c.c. of this specimen of human serum.

Toxin 187 was chosen as the standard toxin in all these experiments as it has been in use at Frankfurt for a number of years, and its m.l.d. is very accurately known. Toxin 590 is of more recent origin than toxin 187 and has been mainly used at Frankfurt for its hæmolytic effect. It is clear from Table I that there is a discrepancy in the *in vitro* reaction of toxin 590. The *in vitro* test suggests that toxin 590 is considerably stronger than is indicated by the Frankfurt figure for its m.l.d.

TABLE I.—In vitro *Titration of Different Toxins using the same Human Serum.*

Toxin in c.c.	0·1	0·09	0·08	0·072	0·064	0·056	0·05	0·044	0·04	0·036
Saline in c.c.	0·0	0·01	0·02	0·028	0·036	0·044	0·05	0·056	0·06	0·064
Toxin in m.l.d	0·25	0·225	0·2	0·18	0·16	0·14	0·125	0·11	0·1	0·09
Human serum in c.c.	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2
Toxin 187	+++	++	+	±	0	0	0	0	0	0
,, 377	+++	+++	++	+	±	0	0	0	0	0
,, 484	+++	++	+	±	0	0	0	0	0	0
,, 590	+++	+++	+++	+++	+++	+++	++	+	±	0

TABLE II.—In vitro *and* in vivo (*Guinea-pig Skin*) *Titration of Toxin* 187 *and Toxin* 590.

Toxin in m.l.d.	0·25	0·225	0·2	0·18	0·16	0·14	0·125	0·11	0·1	0·09	0·08
Human serum in c.c. (*in vitro* test)	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2	0·2
Toxin 187 :											
In vitro	+++	++	+	±	0	0	0	..	..	..	..
In vivo	N.	N.	N.	i.	i.	0	0	..	..	..	..
Toxin 590 :											
In vitro	..	..	..	..	+++	+++	++	+	±	0	0
In vivo	..	..	..	..	N.	N.	N.	N.	N.	i.	0

N. = necrosis ; i. = inflammatory reaction. *In vivo* test read at 48 hours.

It was thought that if toxins 187 and 590 were titrated in the skin of the same guinea-pig, this would be an accurate method of comparing their toxicities for the animal. Accordingly these two dried toxins were dissolved in saline, so that each solution contained 2·5 m.l.d. in 1·0 c.c., and then each solution was titrated *in vitro* with human serum, and *in vivo* by intradermal injection into the skin of the same guinea-pig. Each amount of toxin was injected in a volume of 0·1 c.c. The result of the comparison is shown in Table II, and it will be seen that, as judged by the skin reaction in the guinea-pig, the solution of toxin 590 is twice as toxic as the equivalent solution of toxin 187, and even a little stronger than the *in vitro* reaction indicated.

Since it might be argued that the comparative intradermal test in the guinea-pig is not an accurate measure of the m.l.d. for mice, the latter was investigated directly. Each of four amounts of toxin 590, viz. 0·8 mg., 0·6 mg., 0·5 mg., 0·4 mg., was injected intravenously into 20 mice of weights between 17 g. and 19 g. The result of this *in vitro* titration of toxin 590 is seen in Table III. The experiment was deemed to end 48 hours after the toxin injection. In this experiment the m.l.d. of toxin 590 is slightly more than 0·5 mg., and this compares satisfactorily with the calculated m.l.d. in the *in vitro* test, which is approximately 0·55 mg.

TABLE III.—In vivo *Titration of Toxin* 590 *by Mouse Test.*

Number of mice in series.	Dose of toxin 590.	Survivors at 24 hours.	Survivors at 48 hours.
20	0·8 mg.	0	..
20	0·6 ,,	1	0
20	0·5 ,,	6	1
20	0·4 ,,	14	7

As it was obviously preferable to work with dried toxins about which considerable information was available, comparatively little work was done on fluid toxins prepared in this laboratory, but here again several tests suggested that the *in vitro* and *in vivo* tests ran parallel.

There is some variation between the susceptibility of various human sera to the same toxin, as can be seen by referring to Table IV and Table V. This emphasizes the necessity of using the same human serum when comparing any toxin with the standard toxin. These tables also show the same discrepancy between the standard toxin 187 and toxin 590, whatever human serum is used for the comparison.

Reference to the minimal hæmolytic doses of the four dried toxins shows that the *in vitro* activity of the toxins of *Cl. welchii* (Type A) on human serum bears no apparent relation to the hæmolytic power of the toxin.

c. *Antitoxin Titration.*

For this phase of the work, there were available the standard antitoxic serum (Frankfurt) containing 50 antitoxin units per c.c.; 4 other antitoxic sera from the Behringwerke containing respectively 400, 300, 150 and 100

TABLE IV.—In vitro *Titration of Toxin* 187 *using several Normal Human Sera.*

Toxin 187 in m.l.d. / Human serum in c.c.	0·22 / 0·2	0·2 / 0·2	0·18 / 0·2	0·16 / 0·2	0·14 / 0·2	0·12 / 0·2	0·11 / 0·2	0·1 / 0·2	0·09 / 0·2	0·08 / 0·2
Human serum No. 22	+++	+++	+++	+++	+++	+++	++	+	±	0
,, ,, 29	+++	+++	+++	++	+	±	0	0	0	0
,, ,, 40	+++	++	+	±	0	0	0	0	0	0
,, ,, 52	+++	+++	+++	++	+	±	0	0	0	0

TABLE V.—In vitro *Titration of Toxin* 590 *using several Normal Human Sera.*

Toxin 590 in m.l.d. / Human serum in c.c.	0·1 / 0·2	0·09 / 0·2	0·08 / 0·2	0·072 / 0·2	0·065 / 0·2	0·06 / 0·2	0·055 / 0·2	0·05 / 0·2	0·045 / 0·2	0·04 / 0·2
Human serum No. 22	+++	+++	+++	+++	+++	+++	++	+	±	0
,, ,, 29	+++	+++	+++	+++	++	+	±	0	0	0
,, ,, 40	++	+	±	0	0	0	0	0	0	0
,, ,, 52	+++	+++	+++	++	+	±	0	0	0	0

antitoxin units per c.c.; and 6 antitoxic sera (Wellcome), containing respectively 525, 800, 700, 750, 800 and 750 units. Each of these antitoxins was diluted so that 1·0 c.c. of each contained 1 antitoxin unit. Thus the standard antitoxin was diluted 1/50. Each of the antitoxin dilutions was then titrated against a constant quantity of toxin (2 m.l.d.) and a constant quantity (0·1 c.c.) of human serum. The result of this titration is seen in Table VI. If the *in vitro* and mouse tests run parallel the neutralization end-points shown in the table should all be the same. It will be seen that with 5 antitoxins the neutralization end-point is the same as that of the standard antitoxin, with 4 antitoxins the deviation is approximately 10 per cent. (which is perhaps no greater than could be accounted for by experimental errors in each test), and with 1 antitoxin (Behring 273) the deviation is a little greater than 10 per cent. The correlation between the *in vitro* and the *in vivo* tests is thus shown to be reasonably good.

TABLE VI.—In vitro *Titration of Antitoxins using Standard Toxin* 187.

Units of antitoxin.	0·055	0·05	0·045	0·04	0·036	0·032
m.l.d. of toxin 187	2	2	2	2	2	2
Human serum in c.c.	0·1	0·1	0·1	0·1	0·1	0·1
Standard antitoxin:						
No. 25 (50 units)	0	0	++	+++	+++	+++
Behring antitoxin:						
No. 261 (400 units)	0	0	++	+++	+++	+++
No. 268 (300 ,,)	0	0	++	+++	+++	+++
No. 272 (150 ,,)	0	0	0	++	+++	+++
No. 273 (100 ,,)	0	0	0	0	++	+++
Wellcome antitoxin:						
No. 3269 (525 units)	0	0	0	++	+++	+++
No. 3755 (800 ,,)	0	0	++	+++	+++	+++
No. 3761 (700 ,,)	0	0	++	+++	+++	+++
No. 3762 (750 ,,)	0	0	++	+++	+++	+++
No. 3763 (800 ,,)	0	0	0	++	+++	+++
No. 3766 (750 ,,)	0	0	0	+++	+++	+++

In order to determine whether the neutralization end-point was affected by the particular toxin used, the standard antitoxin and the Behring antitoxins were diluted as before and tested against the three other dried toxins previously mentioned. The result of these titrations is shown in Tables VII, VIII and IX. By comparing the results in Tables VI, VII, VIII and IX it will be seen that the end-point of the four Behring antitoxins relative to the standard antitoxin is approximately the same, irrespective of the toxin used in the test.

TABLE VII.—In vitro *Titration of Antitoxins using Toxin* 377.

Units of antitoxin	0·065	0·06	0·055	0·05	0·045	0·04
m.l.d. of toxin 377	2	2	2	2	2	2
Human serum in c.c.	0·1	0·1	0·1	0·1	0·1	0·1
Standard antitoxin 25	0	+	+++	+++	+++	+++
Behring antitoxin 261	0	+	+++	+++	+++	+++
,, ,, 268	0	+	+++	+++	+++	+++
,, ,, 272	0	0	++	+++	+++	+++
,, ,, 273	0	0	0	+	+++	+++

TABLE VIII.—In vitro *Titration of Antitoxins using Toxin* 484.

Units of antitoxin m.l.d. of toxin 484 Human serum in c.c.	0·045 2 0·1	0·04 2 0·1	·0036 2 0·1	0·032 2 0·1	0·028 2 0·1	0·025 2 0·1
Standard antitoxin 25	0	+	+++	+++	+++	+++
Behring antitoxin 261	0	+	+++	+++	+++	+++
,, ,, 268	0	+	+++	+++	+++	+++
,, ,, 272	0	0	+	+++	+++	+++
,, ,, 273	0	0	0	+	+++	+++

TABLE IX.—In vitro *Titration of Antitoxins using Toxin* 590.

Units of antitoxin m.l.d. of toxin 590 Human serum in c.c.	0·08 2 0·1	0·072 2 0·1	0·065 2 0·1	0·06 2 0·1	0·055 2 0·1	0·05 2 0·1
Standard antitoxin 25	0	+	+++	+++	+++	+++
Behring antitoxin 261	0	+	+++	+++	+++	+++
,, ,, 268	0	+	+++	+++	+++	+++
,, ,, 272	0	0	++	+++	+++	+++
,, ,, 273	0	0	0	++	+++	+++

The amount, however, of the standard antitoxin necessary to neutralize 2 m.l.d. of each toxin shows some variation. This variation in the neutralization end-point with different toxins in the *in vitro* test is paralleled by the neutralization of different toxins by antitoxins, as measured by the reaction in the skin of a guinea-pig (Table X).

In any comparison between the neutralizing power of the standard antitoxin and the neutralizing power of other antitoxins, the same toxin should be used. The neutralization test *in vitro* has been repeated several times with different human sera, with consistently uniform results.

An experiment was designed to determine whether the neutral point in the *in vitro* test corresponded to the neutral point *in vivo*. Using a certain toxin and a certain antitoxin it was found that 0·05 of an antitoxin unit was just able to neutralize 2 m.l.d. of the toxin in the *in vitro* reaction. On the principle of multiple proportions, 0·25 of an antitoxin unit should be able to neutralize 10 m.l.d. of toxin, and 0·225 of an antitoxin unit when added to 10 m.l.d. of toxin should leave 1 m.l.d. of toxin free. These two mixtures, viz.,

A. 0·25 units of antitoxin + 10 m.l.d. of toxin.
B. 0·225 units of antitoxin + 10 m.l.d. of toxin,

were injected into mice. The mouse given mixture A survived, the mouse given mixture B died. Small though this experiment is, it suggests that the *in vitro* neutral point approximates to the *in vivo* neutral point.

Four antitoxic sera for experimental use were received from a third laboratory. The *in vitro* titration agreed with the indicated unitage in two of these sera, there was a discrepancy of 25 per cent. in the third serum, and

TABLE X.—In vitro *and* in vivo (*Guinea-pig Skin*) *Titrations of Standard Antitoxin No.* 25 *with Toxin* 187 *and Toxin* 590.

Units of standard antitoxin No. 25	0·08	0·072	0·065	0·06	0·055	0·05	0·045	0·04	0·036	0·032	0·028
m.l.d. of toxin	2	2	2	2	2	2	2	2	2	2	2
Human serum in c.c. (*in vitro* test).	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1
Toxin 187 :											
In vitro	..	..	..	..	0	0	++	+++	+++	+++	+++
In vivo	..	..	..	..	0	0	0	i.	i.	N.	N.
Toxin 590 :											
In vitro	0	+	+++	+++	+++	+++	+++	..	..	..	..
In vivo	0	0	i.	i.	N.	N.	N.	..	..	..	..

N = necrosis ; i. = inflammatory reaction. *In vivo* test read at 48 hours.

in the fourth serum the discrepancy was more than 100 per cent. The latter serum was titrated by the intradermal method, and the result of this *in vivo* titration agreed with the *in vitro* titration.

D. Cl. welchii *Types B, C and D.*

It has already been pointed out that cultures of *Cl. welchii* Types B, C and D produce the visible change in human serum characteristic of Type A, and that the change is inhibited by Types A, B, C and D antitoxin. Since it is known that all these four types of *Cl. welchii* are capable of producing the Type A lethal toxin, it is probable that the change in the human serum produced by Types B, C and D is due to this Type A lethal toxin, and that the inhibition of the change in human serum by antitoxin Types B, C and D is due to the Type A antitoxin which they contain in addition to their own specific type antitoxin. This contention is borne out by an experiment in which each of these antitoxins were titrated against 2·0 m.l.d. of the Type A standard toxin. Table XI shows that the Type B antitoxin contains roughly 50 Type A antitoxin units per c.c., the Type C antitoxin about 5 Type A antitoxin units per c.c., and the Type D antitoxin about 25 Type A antitoxin units per c.c., as measured by the *in vitro* test. As there is no *in vivo* check on these figures, they are only presumptive.

TABLE XI.—In vitro *Titration of* Cl. welchii *Types B, C and D Antitoxins against* Cl. welchii *Type A Toxin.*

Antitoxin in c.c.	0·1	0·05	0·02	0·01	0·005	0·002	0·001	0·0005
m.l.d. of toxin 187	2	2	2	2	2	2	2	2
Human serum in c.c.	0·1	0·1	0·1	0·1	0·1	0·1	0·1	0·1
Antitoxin of Type B	0	0	0	0	0	0	0	+++
,, ,, C	0	0	0	0	+++	+++	+++	+++
,, ,, D	0	0	0	0	0	0	+	+++
Normal sheep serum	+++	+++	+++	+++	..	..	..	..

Attempts were made to prepare toxins in this laboratory from Types B, C and D. All these toxins were lethal to mice, death occurring within one hour after injection of the toxin, but in only one case (a toxin prepared from a Type D strain) did the toxin change the human serum. The change was inhibited by Type A antitoxin.

SUMMARY.

1. A reaction is described between the lethal toxin of *Cl. welchii* (Type A) and normal human serum in which the serum becomes opalescent. When the opalescent serum is centrifuged, a fatty material rises to the surface.

2. The reaction is specifically inhibited by *Cl. welchii* antitoxin.

3. The reaction can also be observed if *Cl. welchii* of any type (A, B, C or D) is grown in a mixture of normal human serum and broth. This cultural

reaction has been shown to be specific for *Cl. welchii*, and it is specifically inhibited by *Cl. welchii* antitoxin.

4. The lethal toxin of *Cl. welchii* (Type A) can be titrated by means of the reaction, the activity of the toxin in bringing about the change in human serum running parallel to its toxicity for mice.

5. *Cl. welchii* (Type A) antitoxin can likewise be titrated, the inhibiting action of the antitoxin *in vitro* running parallel to its neutralizing action *in vivo*.

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A PHYSICAL AND CHEMICAL EXAMINATION OF VACCINIA VIRUS.

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VARIOUS techniques have been described by which comparatively large quantities of the elementary bodies which are the causal agents of vaccinial lesions can be obtained from the infected tissue (Ledingham, 1931 ; Craigie, 1932).

The criterion of purity of such preparations was at first almost wholly immunological, a suspension being considered " pure " if it was specifically agglutinated by a vaccinial anti-serum and not agglutinated by an antiserum to the tissues of the host. Hughes, Parker and Rivers (1935) showed the presence of protein, fat and carbohydrate in suspensions of elementary bodies conforming to this requirement. More recently the degree of uniformity in particle size determined by centrifugal analysis has been emphasized as an accessory criterion of purity (Beard, Finkelstein and Wyckoff, 1937 ; Amies, 1938 ; Pickels and Smadel, 1938).

Smadel, Rivers and Pickels (1939) take as their criterion of purity the ratio between the total number of particles and the number of infective units.

For their own suspensions they obtain an average ratio of 4·2 to 1 ; for the suspensions used by Macfarlane and Salaman (1938) they calculate that the ratio was 1000 : 1. They therefore conclude that the latter suspensions contained an excessive amount of "impurity", and consider that such suspensions are not suitable for investigations on the nature of vaccinia virus. This criticism, which is applicable also to the material used in the present work, is one with which we cannot agree.

Firstly, since for the determination of the infective titre different methods, both admittedly liable to considerable error, were used by the two groups of workers, it is possible that the difference in infective titre of suspensions prepared by essentially the same technique is more apparent than real ; it is hoped to check this point by direct comparison of the two methods.

The more important point at issue is the reality behind the word "impurity". The American authors consider that in their own case the non-infective material is inactive virus, but that in the case of Macfarlane and Salaman it is an unspecified impurity. There is no *a priori* reason nor any experimental evidence for this distinction ; in the case of our own suspensions there is considerable support for the view that the non-infective material cannot be distinguished except by infectivity tests from the active virus.

The chief evidence of homogeneity in the virus suspensions we have used is the high degree of uniformity in particle size. The limits of variation in this size are of quite a different order from those which arise from the technique of differential centrifugation. It is also extremely unlikely that the high yield of homogeneous virus can be due either to the presence of large numbers of particles of rabbit tissue, or of any hypothetical substance which arises from the action of the virus on the rabbit tissue, since from rabbits inoculated with inhomogeneous virus, which gives rise to similar but even more inflammatory lesions, only an insignificant amount of homogeneous virus suspension is obtained by the usual technique (Beard *et al.*, 1937 ; Amies, 1938). The negative results obtained by Macfarlane and Salaman in enzymic tests confirm the absence of rabbit tissue, since the virus suspensions did not contain certain enzymes readily demonstrable in clarification deposits.

The physical and chemical data obtained by various workers, using suspensions in which the ratio of total to infective particles presumably varies within wide limits, are in good agreement ; that is to say, the so-called impurities are indistinguishable by the physical and chemical methods so far used. It is known also that plant viruses, as well as enzymes, can be inactivated by a variety of means, without any detectable change in their physical or chemical properties. We therefore feel justified in assuming that our suspensions consist essentially of active and an unspecifiable amount of inactive virus. Physical and chemical data derived from investigations of virus particles showing a high degree of physical homogeneity are now presented.

METHODS.

Microscopic examination.

For visual examination mercury green light (5460 Å) and a Beck dark-field condenser with 2 mm. oil-immersion objective was used. Photomicrographs

were taken with a Bausch & Lomb 1·7 mm. "ultraviolet" objective and mercury violet (3655 Å) light.

Sedimentation analysis.

The Svedberg equilibrium centrifuge was used at a standardized speed of 3900 r.p.m. and a temperature of 20° C. The ultraviolet light absorption method using a band 2300 Å–2700 Å was employed for photography, the visible scattering caused by the virus being too great to permit of the use of any of the refraction methods. Actually the absorption in the ultraviolet must represent a complicated function both of absorption and Tyndall scattering, and this imposes a limitation on the quantitative deductions permissible from the photographs. Exposure times were 5–10 seconds every 8 minutes, using Ilford ordinary plates.

Dry density.

A suspension in dilute salt or buffer solution of about 60 mg. of virus, which had been previously flocculated at pH 4·5, was weighed in a 1·2 ml. pyknometer after equilibrating at 25° C. The contents of the pyknometer were washed out quantitatively with distilled water, frozen, dried over P_2O_5 *in vacuo* and weighed. The pyknometer was again weighed filled with the suspending medium. Small air-bubbles enclosed in the virus floccules were effectively removed by light centrifugation, or partial evacuation of the suspension in a pressure flask.

Chemical analysis.

All analyses are calculated on the dry weight, moisture being determined by the loss of weight on drying in a Pregl drier at 100° C. Phosphorus was estimated colorimetrically by Brigg's method after ashing with H_2SO_4 and perhydrol; nitrogen by the micro-Dumas or, in the case of solutions, by the micro-Kjeldahl technique after incineration with H_2SO_4 and perhydrol; ash, by slow heating in an electric muffle to a final temperature of 700°; cholesterol, by colorimetric estimation after precipitation as the digitonide (Schoenheimer and Sperry, 1934). Total carbohydrate was estimated by the colour developed on heating at 100° for 10 minutes with 10 volumes of 0·2 per cent. orcinol in 66 per cent. sulphuric acid using mannose as standard (Pirie, 1936). The significance of this and other carbohydrate tests is discussed later.

Preparation of homogeneous suspensions by differential centrifugation.

The strain of virus used in these experiments was originally obtained from sheep lymph. It had already been passaged 90 times on rabbit skin by Dr. M. H. Salaman, and in the course of the present work it was subjected to 25 further passages on rabbits without undergoing any apparent change in character. The method of harvesting the virus is essentially that described by Craigie (1932) and modified by Salaman (1937). The skin pulp was shaken mechanically with $M/250$ citric acid-disodium phosphate buffer (pH 7·2), the

tissue debris being deposited by centrifugation and re-extracted twice in the same way. The virus was then deposited from the pooled extracts by centrifugation in an angle centrifuge. The deposit was taken up in fresh buffer solution and washed twice in this way ; finally the suspension was centrifuged at 3000 r.p.m. for half an hour in the horizontal centrifuge, during which time a deposit was formed which sometimes weighed more than the supernatant virus. It has been shown by Beard *et al.* (1937) and confirmed by Amies (1938) that if the virus used as inoculum has undergone a sufficient number of serial passages, the suspensions obtained after this "clarification" consist of particles which appear uniform in size on dark-field examination, and give rise in the analytical ultra-centrifuge to boundaries which are only slightly blurred. The yield of this homogeneous virus varied from 30 mg. to 60 mg. of dry virus per rabbit. A further differential centrifugation of these suspensions on the horizontal centrifuge does not effect a significant improvement in homogeneity. By dark-field examination the clarification deposit consisted of a few bacteria, some hairs, a small quantity of shapeless cell detritus, and a large quantity of densely aggregated virus. Since the latter appeared to represent the major part of the deposit a considerable loss of virus occurs in the clarification process. The infectivity of the deposit is high, but owing to the agglutination of the virus, cannot be accurately estimated.

The virus suspension was kept at 0°. Prior to analysis the virus was flocculated by addition of $M/50$ acetic acid-sodium acetate buffer (pH 4·5), and washed rapidly first with $M/50$ sodium chloride to facilitate removal of phosphate ions and then with distilled water. In this way approximately isoelectric virus of minimum ash content was obtained. The final aqueous washings contained less than 0·002 mg. P, compared with 0·05 to 0·5 mg. P. contained in the deposited virus, according to the quantities handled. The deposit was dried *in vacuo* over P_2O_5 from the frozen state ; the dried material took up from 5–8 per cent. moisture on exposure to air.

Properties of the untreated virus.

The sedimentation pictures obtained from these virus suspensions do not differ in any important detail from those previously published (Pickels and Smadel, 1938 ; Beard *et al.*, 1938). The boundaries appear quite sharp in the first few photographs and in the later ones become blurred. We have noted in a few cases the appearance of multiple boundaries described by the first-named authors and attributed by them to particle aggregation. We obtain a mean sedimentation constant, corrected to water at 20° C., of 5000×10^{-13} for the rather sharp upper edge of the boundary. Values of 4910 and 5400 were obtained by Pickels and Smadel and by Beard, Finkelstein and Wyckoff respectively ; it is not stated whether the latter value refers to the upper edge or to the middle of the boundary.

The content of nitrogen (13·1 per cent.) and phosphorus (0·8 per cent.) was similar to that previously found (Macfarlane and Salaman, 1938). From the dried virus 8 to 10 per cent. of lipoid material could be extracted with ether. This is in agreement with the findings of Hughes *et al.* (1935). Extraction with

alcohol-ether mixtures, however, removed up to 20 per cent. of lipoid material. Tests for carbohydrate and thymonucleic acid (Feulgen reaction) were positive on quantities of 1–2 mg. dry weight. Dry density values are given in Table I, there being some slight variation about a mean value of 1·26.

TABLE I.—*Dry Density of Vaccinia Virus.*

Virus preparation.		Medium.	Density.	Mean.
Native virus	72B	*M*/1000 sodium chloride	1·27	1·26
	88A	,, ,, ,,	1·27	
	88B	*M*/250 citric acid-disodium phosphate	1·27	
	94	,, ,, ,, ,,	1·24	
	101	*M*/100 sodium chloride	1·26	
	102	*M*/250 ,, ,,	1·25	
Ether-extracted virus	101	*M*/50 sodium chloride	1·31	1·31
	103 + 104	Phosphate buffer pH 8·0; μ = ·004	1·29	
	107 + 109	*M*/50 sodium chloride	1·29	
	111 + 115	,, ,, ,,	1·30	
	112	,, ,, ,,	1·33	
	113	,, ,, ,,	1·32	
	116	,, ,, ,,	1·32	
Alcohol-ether extracted virus	111 + 115	*M*/50 sodium chloride	1·35	1·36
	112	,, ,, ,,	1·37	
	116	,, ,, ,,	1·37	

Measurement of particle hydration.

According to various authors (Elford and Andrews, 1936; Elford, 1938; Smadel *et al.*, 1938) the density of the virus in the wet state is 1·16–1·18, indicating the association of 1 volume of water with approximately 2 volumes of dry matter. This view is based on sedimentation data, and experiments have been made to measure the hydration by an independent method. The virus used was suspended in as small a volume of buffer solution as was practicable for accurate measurement. A known volume of this suspension was mixed in a centrifuge tube with a known volume of sucrose or egg albumin solution, an equal volume of buffer solution being similarly treated in a second tube. The virus was deposited by centrifugation and the concentration of sucrose in the supernatant fluid compared with that in the second tube. The concentration is greater in the former in proportion to the effective volume occupied by the virus particles and any associated water into which the sucrose does not diffuse. If diffusion into the virus takes place or if sucrose is concentrated at the surface of the virus, the results will indicate a smaller degree of hydration than actually exists. The concentration of the solute was determined by means of the Zeiss dipping refractometer, using the accessory prism, which enables measurements to be made on 0·1–0·2 ml. of solution. It was found by preliminary washing of the virus and examination of the supernatant fluid, and by nitrogen determinations on the supernatant fluids after addition of the solutions, that no measurable dissociation of material from the virus which might have vitiated the results had taken place before or during contact with the solute.

The results show (Table II) that in the presence of low concentrations of sucrose or egg albumin relatively enormous amounts of water—7 to 8 times the volume of dry virus—are so bound as to be impenetrable to the solute molecules. These results are confirmed by rough measurements of the volume of the deposited virus. In the experiments with dilute solutions of sucrose or egg albumin the volume of the deposit was 6 to 8 times the known volume of dry virus.

TABLE II.—*Data Relating to Measurements of Virus Hydration by Partition Method.*

Dry weight of virus.	Dry volume of virus.	Volume of virus suspension.	Partition solution.			Concentration of solute in supernatant.	Effective volume of hydrated virus.	Volumes H_2O : 1 volume dry virus substance.
			Solute.	Concentration.	Volume added.			
gm.	ml.	ml.		%	ml.	%	ml.	
·0208	·0165	0·424	Sucrose	14·45	0·2	4·90	·034	1·06
·0263	·0206	0·424	,,	4·75	0·2	1·92	·128	5·21
·0190	·0150	0·400	,,	1·86	0·2	0·74	·102	5·80
·0322	·0254	0·424	,,	1·73	0·2	0·80	·192	6·55
·0325	·0256	0·424	Egg albumin	5·29	0·2	2·02	·100	2·90
·0396	·0314	0·600	,, ,,	4·51	0·2	1·52	·210	5·68
·0283	·0223	0·424	,, ,,	1·39	0·2	0·64	·191	7·56

Behaviour towards lipoid solvents.

If a suspension of elementary bodies is shaken with ether less than 1 per cent. of material passes into ethereal solution even with repeated extraction. The infectivity of the suspension is not affected by this procedure (Craigie and Wishart, 1934). Treatment of dried virus with ether, however, removes a quantity of lipoid material varying from 8 to 10·5 per cent. with different preparations, again without loss of infectivity (*cf.* Table III). From samples of any one preparation the same proportion of lipoid material was extracted with benzene as with ether, the whole of the ether or benzene-soluble* fraction being removed in two extractions at room temperature. The amount of lipoid material extracted from the virus was also the same irrespective of whether the virus was in the form of a bulky powder obtained by drying from the frozen state or a tough "skin" obtained by drying at room temperature. The composition of the ether or benzene extracts was rather variable, but consisted almost entirely of acetone-soluble material, including 14 to 20 per cent. of cholesterol. The nitrogen and phosphorus contents were 2–3 per cent. and 0·8–1 per cent. respectively; carbohydrate and protein tests were negative.

Extraction with ether or benzene removed significantly higher amounts of lipoid material (13–18 per cent.) from the clarification deposits referred to above than from the homogeneous virus; the extracts contained cholesterol, nitrogen and phosphorus in substantially the same ratio as before but in

* Throughout the paper "ether-soluble material" means material extracted from the virus with ether alone, "alcohol-soluble" that extracted with alcohol or alcohol-ether mixtures. The lipoid material extracted was soluble both in alcohol and ether.

rather lower percentage. Examination of successive deposits from a suspension of virus subjected to repeated clarifications showed that both the amount of the deposit and its content of ether-soluble material decreased until the lipoid content was nearly the same as that of the supernatant virus. For example, in one case the first clarification gave a deposit which weighed 57 mg. and contained 10·5 per cent. of ether-soluble material; a second clarification of the supernatant suspension gave 23 mg. of deposit containing 6·6 per cent. of ether-soluble material, and left in the supernatant suspension 128 mg. of virus containing 5·3 per cent. of ether-soluble material. It seemed probable that the presence of this ether-soluble material facilitated aggregation of the virus, and that considerable quantities of virus discarded in the clarification deposits might be made available by removal of the lipoid material and resuspension of the virus.

Resuspension of flocculated virus.

It has been generally believed that flocculation of vaccinia virus is an irreversible process. If the floccules are mechanically dispersed by shaking or pipetting, the virus is invariably deposited again from the resulting suspension after standing for a few hours. If the suspension is examined immediately in the ultracentrifuge, most of the virus sediments at an abnormally high rate and the multiple boundaries already referred to are seen. This has been our consistent experience with the untreated virus.

The dry ether-treated virus behaves in this way if dispersed in buffer solution. If however it is first washed with distilled water to remove salts and then cautiously treated with $M/100$ NaOH until the pH is 9·0 to 9·5 a dilute but stable suspension is obtained. By repeated shaking with small quantities of alkali almost all the virus can be recovered in stable milky suspension, and may be deposited and resuspended in the usual buffer. It has now lost its pronounced tendency to stick to glass and the accurate pipetting of samples is therefore easier.

It is clear that the agglutination which accompanies drying of the virus is in fact reversible. Though the virus can withstand exposure to a pH of 9·5 the method is tedious and requires careful control of the pH. In order to achieve the resuspension without exposing the virus to such a high pH mechanical dispersion devices were tried. The simple form of tubular grinder shown in Fig. 1 and devised by Seifried (1939) for disintegrating soft tissues proved strikingly successful.

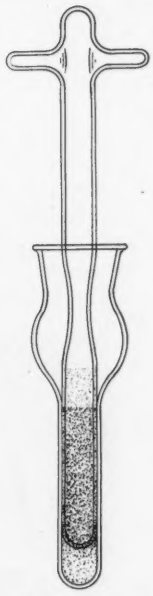


FIG. 1.—Seifried tissue grinder used for resuspending the dried virus.

The plunger must be ground to fit closely. The dry virus is placed in the bottom of the tube, covered with 5 ml. of phosphate buffer of pH 8·0 and ionic strength $\mu = \cdot 004$, and forced up and down in

the space between the plunger and the wall of the tube. As in the alkali method only small quantities of virus can be resuspended at one treatment, the emulsion being centrifuged and the deposit returned to the grinder with a fresh lot of buffer. In this way up to 70 per cent. of the dry virus may be resuspended according to the clearance in the grinder. The residue consists of small aggregates which pass through the annular orifice of the grinder without suffering any shear.

Both the alkali method and the use of the grinder are less successful with dried virus which has not been extracted with ether, the presence of the ether-soluble material appearing to enhance the cohesion of the virus aggregates; this is in agreement with the evidence already given that spontaneous aggregates of virus have a higher lipoid content than the single virus particles left in the supernatant. Virus dried from the frozen state is much easier to resuspend than virus dried at room temperature.

Properties of ether-extracted resuspended virus.

Intradermal inoculation tests on homogeneous suspensions of ether-extracted virus showed that no significant change in infectivity had taken place (Table III).

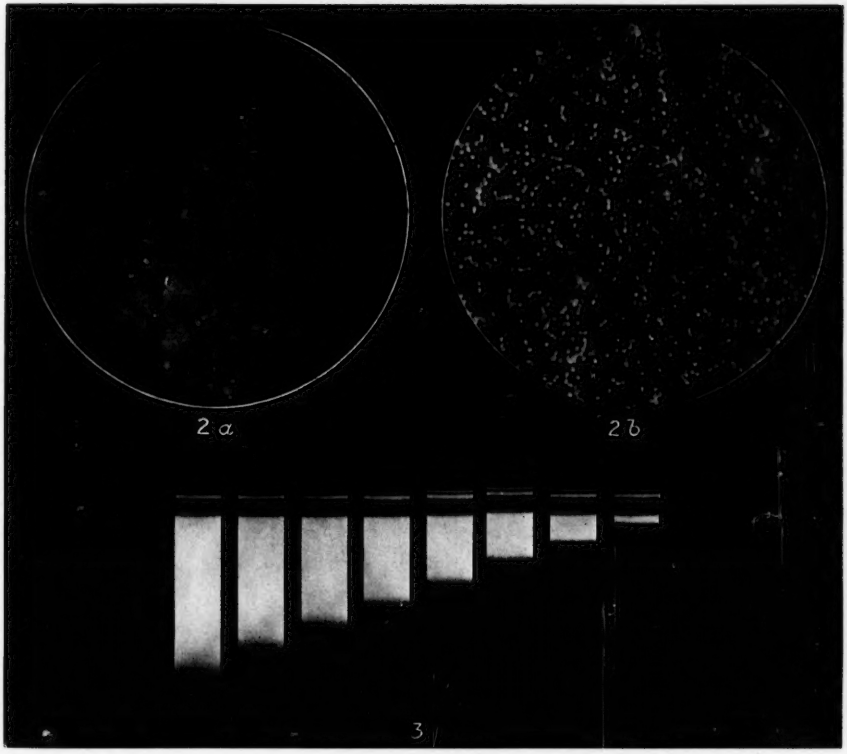
TABLE III.—*Effect of Drying and Lipoid Extraction of Vaccinia.*

All suspensions diluted to 2·5 mg. dry virus per ml. and 0·1 ml. used for intradermal inoculation.

Virus.	Treatment of virus.	Rabbit.	Titre.
98	Untreated	119	10^{-6}
,,	Frozen, dried and re-shaken with buffer to original volume	,,	10^{-6}
,,	Frozen, dried, extracted with benzene and re-shaken with buffer to original volume	,,	10^{-5}
,,	Frozen, dried, extracted with benzene and resuspended with alkali at pH 9–9·5	,,	10^{-5}
98 and 99	Frozen, dried and re-shaken with buffer	123	10^{-5}
,,	Frozen, dried, extracted with benzene and re-shaken with buffer	,,	10^{-5}
98	Frozen, dried, extracted with benzene, resuspended with alkali twice centrifuged and suspended in buffer	,,	10^{-5}
104	Frozen, dried and re-shaken with buffer	130	10^{-8}
,,	Frozen, dried, extracted with benzene and re-shaken with buffer	,,	10^{-7}
,,	Dried at room temperature, re-shaken with buffer	,,	10^{-6}
,,	Dried at room temperature, extracted with benzene and re-shaken with buffer	,,	10^{-6}
106	Frozen, dried and extracted with benzene	135	10^{-4}
,,	,, ,, and extracted with ether	,,	10^{-6}
118	,, ,, and re-shaken with buffer	136	10^{-6}
,,	,, ,, extracted with anhydrous alcohol-ether and re-shaken with buffer	,,	10^{-2*}

* A small atypical lesion.

Fig. 2*b* is a dark-ground photomicrograph of the virus after ether-extraction



FIGS. 2*a* and 2*b*.—Dark-ground photomicrographs of (*a*) flocculated and (*b*) redispersed elementary bodies.

FIG. 3.—Sedimentation photographs of dried, ether-extracted and re-suspended virus using U. V. light-absorption technique. Photographs at 6-minute intervals. Force 1000 xg.

and resuspension by grinding; the elementary bodies have a normal appearance. For comparison, there is shown in Fig. 2*a* a photomicrograph of a large floccule, formed by drying the virus; this dried virus was subsequently effectively redispersed by grinding. Sedimentation pictures of suspensions of the ether-extracted virus (Fig. 3) show that the amount of boundary spreading is rather less than in the original virus suspension. The sedimentation constant is not significantly different from that of the untreated virus. Pyknometric measurements of dry density gave a mean value of 1·31 (Table I), which is significantly higher than the value for the unextracted virus.

For chemical analysis the resuspended virus was flocculated at pH 4·5, washed with *M*/50 NaCl and then with water until the washings contained no appreciable amounts of phosphate, and dried from the frozen state. The following analytical figures were obtained for three samples prepared in this way: N (Dumas) 14·5, 14·9, 14·7 per cent.; P 1·0, 0·7, 0·8 per cent.; carbohydrate 3·3 per cent. (as mannose). The orcinol tests for pentose and the Feulgen reaction were positive, indicating the presence of thymo-nucleic acid.

Treatment of infective, ether-extracted virus with alcohol.

If the ether-treated virus, after resuspension and drying, is again treated with ether or benzene, only the smallest trace of lipoid material is extracted. Treatment with alcohol-ether (1 : 1) mixtures, however, removed 8·4 to 10 per cent. of the substance and the composition of this alcohol-soluble material differed considerably from that of the ether-soluble fraction described above. No cholesterol was present, and the P and N contents were appreciably higher, averaging 2·3 and 3·7 per cent. respectively. The extract contained traces of carbohydrate but no protein. The P content would correspond to a lecithin content of 55 per cent. but the constituents have not been characterized.

The dry virus powder after extraction with ether-alcohol does not wet spontaneously. Nevertheless, treatment in the grinder in the manner already described gave rise to a milky suspension with a short stability period. Dark-ground examination showed particles with reduced light-scattering capacity but otherwise similar in appearance to elementary bodies. In the centrifuge the boundaries were so diffuse as to be almost undetectable and no significant determinations of sedimentation constant could be made. The mean dry density was 1·36; the figures for different virus preparations show some variation which is just outside the limits of experimental error (Table I).

Intradermal inoculation of the resuspended virus after alcohol-ether extraction showed that the infectivity had been almost completely destroyed. Slight reactions occurred at the site of inoculation of some of the higher concentrations, but these were atypical and could not be regarded as genuine vaccinial lesions. The loss of infectivity occurred even when scrupulous care was taken to carry out the alcohol treatment in strictly anhydrous conditions. This is in contrast to the finding of Dearing (1934) that his mouse strain of vaccinia virus could be treated with anhydrous alcohol without loss of infectivity.

Analytical figures for various samples of the alcohol-ether treated virus are shown in Table IV.

TABLE IV.

Preparation.	Per cent. composition. N.	P.	Carbohydrate (as mannose).	Ash.
112	15·1	0·78	2·8	2·3
113	15·6	0·70	3·1	2·7
111 + 115	15·1	0·76	3·1	2·3
116	15·5	0·71	..	..

The pentose content was estimated by comparison of the colour developed by heating with 1 volume of 0·15 per cent. orcinol in concentrated HCl for 6 minutes at 100°, using yeast or thymonucleic acid as standard. In these conditions, 1 ml. solution containing 0·1 mg. ribose gives a pale blue colour, while 0·4 mg. desoxyribose (as thymonucleic acid) gives no colour. If, however, the test is carried out in the presence of ferric chloride (0·1 volume 0·27 per cent. solution) as little as 0·02 mg. desoxyribose gives a perceptible green blue colour, while the colour developed by ribose is only slightly increased. In the absence of $FeCl_3$ the virus material (3·0 mg./ml.) gave no blue colour on heating with orcinol-HCl, while in the presence of $FeCl_3$ the green colour developed was roughly equivalent to a thymonucleic acid content of 3·4 per cent. In the presence of a large excess of protein, which tends to give a grey colour to the solution or to amyl alcohol extracts and also to absorb the pigment, the values are only approximate. With specimens of higher nucleic acid content, such as 20 per cent. or over, the estimation is fairly satisfactory.

The values for carbohydrate given in Table IV were obtained by the orcinol-sulphuric acid method and are arbitrarily expressed as mannose. The colour development in this method per unit weight of sugar varies widely under identical conditions for different sugars. For instance, the colour developed by amounts of ribo- and thymo-nucleic acids containing 0·10 mg. sugar equalled 0·08 and 0·022 mg. mannose respectively, that by 0·10 mg. glycuronic acid equalled 0·066 mg. mannose, while glucosamine gave no colour at all. If the carbohydrate reaction given by the virus is assumed to be due entirely to thymonucleic acid, the calculated amount of the latter (34 per cent.) is far higher than the maximum amount possible on the P content (8 per cent.), and also than the amount indicated by the pentose tests. It is presumed therefore that the virus contains both thymonucleic and some other carbohydrate complex. Tests for glucosamine on material previously hydrolysed for 3 hours at 100° in 5 *N* HCl (Sørensen, 1938) were positive, showing the presence of about 1 per cent. of glucosamine-residues.

Modified technique for preparation of virus suspensions.

It is concluded from the foregoing experiments that at present the ether treated and resuspended particle must be assumed to be the virus proper. For many purposes the technique of preparation may therefore be advan-

tageously altered in the following way: The suspension prior to the final clarification is dried from the frozen state; after extraction with ether, the virus is resuspended with the aid of the grinder and clarified by spinning horizontally for half an hour at 3000 r.p.m. This gives a homogeneous suspension containing substantially larger quantities of virus than the usual procedure. In order to distinguish such suspensions from those which have been prepared in the manner described by Craigie and which therefore still contain non-specific lipoid material the latter will be arbitrarily referred to as *native* virus.

Disruption of the virus particle by treatment with Na_2CO_3.

Preliminary experiments showed that on treatment of virus suspensions with NaOH or Na_2CO_3 for short periods at a pH above 10 a large part of the material remained in the solid state, but measurable quantities of nitrogen and phosphorus passed into solution. The experiments were repeated on a larger scale in attempts to characterize the fractions obtained.

Experiment A (Table V).—Native virus was washed in rapid succession with $M/50$ NaCl and then with water, dried and extracted with alcohol-ether (1 : 1). The material can therefore be considered as being free from lipoid material but may still contain slowly-dissociable antigen of the kind described by Craigie and Wishart (1936). 86 mg. of dry material were finely ground with 3·7 ml. water in a centrifuge tube; Na_2CO_3 solution was added to a final concentration of 1 per cent. and the suspension was centrifuged after $2\frac{1}{2}$ hours at 20°. The clear solution was removed quantitatively to another centrifuge tube and adjusted to pH 5·65 with acetic acid and sodium acetate buffer; the resulting precipitate was separated by centrifuging and re-dissolved in dilute sodium carbonate solution. The residue from the first Na_2CO_3 extraction was suspended overnight in 1 per cent. carbonate at 20°, the solution separated and the residue suspended in water.

Experiment B (Table V).—A homogeneous suspension of native virus in phosphate buffer of pH 8·0 and $\mu = 0{\cdot}004$ was first gently shaken for 5 hours at 20° on a slowly rotating wheel (with the object of removing dissociable antigen). The virus was then deposited in the angle centrifuge and redeposited (in a flocculated state) three times from $M/10$ acetate buffer of pH 4·5, once from $M/10$ NaCl and twice from water; it was then dried and extracted with ether. The first two supernatant fluids contained nitrogen and carbohydrate, but no appreciable quantities of nucleic acid; the later washings contained only traces of carbohydrate and small quantities of organic P and N. 235 mg. dry virus was resuspended in 9·5 ml. water by the use of the grinder and treated with sodium carbonate as shown (Table V).

The results of the two experiments are in substantial agreement, but the figures are not directly comparable, since those for Experiment B include the P and N present in the alcohol-soluble fraction. On short exposure to 1 per cent. sodium carbonate practically all the nucleic acid, 40 per cent. of the carbohydrate, 50 per cent. of the non-lipin P and 20 per cent. of the nitrogen passed into solution. On repeated extraction with carbonate the residue passed gradually into solution. Physical examination of these later extracts,

TABLE V.—*Disintegration of Vaccinia Virus in Alkaline Medium.*

Fraction.	mg. N.	mg. P.	mg. Carbohydrate (as mannose).	mg. Nucleic acid (by pentose).	Percentage distribution of total amount recovered. N.	Percentage distribution of total amount recovered. P.	Percentage distribution of total amount recovered. Carbohydrate.	Percentage distribution of total amount recovered. Nucleic acid.
A. Alcohol-ether treated virus.								
(1) S.F.* after 2½ hrs.' treatment with 1 per cent. Na_2CO_3 at 20° acidified to pH 5·65:								
(*a*) Solution	1·04	0·125	0·77	0·16	16·2	49·5	44·2	80
(*b*) Precipitate	0·80	0·082	0·18	1·00				
(2) S.F. after further 16 hrs. in 1 per cent. Na_2CO_3 at 20°	0·77	0·036	0·20	0·30	6·8	8·5	9·3	20
(3) Insoluble residue	8·72	0·175	1·00	*nil*	77·0	42·0	46·5	0
Total recovered	11·3	0·418	2·15	1·46	..	..	..	..
Original material	12·9	0·61	2·8	2·0 approx.	..	..	..	..
B. Ether treated virus.								
(1) S.F. after ½ hr. in 1 per cent. Na_2CO_3 at 20° acidified to pH 5·65:								
(*a*) Solution	3·10	0·464	2·3	2·1	15·1	33·2	36·8	63
(*b*) Precipitate	1·42	0·13	0·31	0·90				
(2) S.F. after 2nd ½ hr. in 1 per cent. Na_2CO_3 acidified to pH 5·65:								
(*a*) Solution	1·00	0·09	0·38	0·62	4·7	6·7	6·5	21
(*b*) Precipitate	0·42	0·03	0·08	0·40	..	..	..	..
(3) S.F. after further 24 hrs. in 1 per cent. Na_2CO_3	4·30	0·15	0·54	0·8 approx.	14·3	8·4	7·6	16
(4) ,, ,, ,, ,,	2·00	0·06	0·40	..	6·7	3·3	5·6	..
(5) Residue from (4) dried and extracted with alcohol-ether:								
(*a*) Alcohol ether extract	0·27	0·31	..	..	0·9	17·3	..	..
(*b*) Lipin-free residue	17·55	0·55	3·1	..	58·5	30·7	43·6	..
Total recovered	30·06	1·79	7·1	4·8 approx.	..	..	..	..
Original material	32·6	1·99	6·6	6·0 approx.	..	..	..	..

* Supernatant fluid.

which had a characteristic blue opalescence, showed that they contained particles of widely different sizes; on adjusting the pH to 4·6 (Table VI) the total substance was more or less evenly distributed between precipitate and solution. Judging from the limited number of analyses made the later extracts had all the same chemical composition.

Fractionation of the first Na_2CO_3 extract in Experiment A by acidification to pH 5·6 gave a precipitate containing most of the nucleic acid and a solution rich in carbohydrate and organic phosphorus, whereas in Experiment B the nucleic acid was present both in precipitate and solution. The only

TABLE VI.—*Fractionation of Na_2CO_3 Extracts by Precipitation at pH* 4·6.

Fraction.	N. mg.	P. mg.	Carbohydrate. mg.	Protein (N × 6·25). mg.
B (3) acidified to pH 4·6 :				
(*a*) Solution . .	1·40	·044	0·18	8·75
(*b*) Precipitate .	0·93	·032	0·18	5·80
B (4) acidified to pH 4·6 :				
(*a*) Solution . .	0·56	·016	0·14	3·50
(*b*) Precipitate .	0·70	·021	0·16	4·4

obvious reason for this irregularity is that the preliminary treatment of the virus was different. Tests for glucosamine on the solution after hydrolysis in HCl were negative; by contrast the test was positive on samples of the residue, but the amounts tested were in both cases rather small (containing 0·1 mg. carbohydrate).

Treatment with sodium carbonate may therefore be interpreted as having two effects: a rapid transference of nucleic acid and certain other constituents, notably part of the carbohydrate, into solution, overlapped and followed by a slow disruption of the residue (which contains carbohydrate and non-lipin P) into fragments of varying size but approximately similar chemical composition. It may be presumed that at least part of the nitrogen present in the first Na_2CO_3 extracts was contained in protein fragments similar to those in later extracts, and it is only to be expected that a protein precipitate at pH 5·6 would carry down the nucleic acid. There is, however, no evidence that the nucleic acid is liberated from the main body of the particle as a nucleoprotein of definite composition. It may be noted that the presence or absence of lipoid material does not apparently affect the course of this disruption.

Purification of nucleic acid fractions.

Attempts to separate nucleic acid in pure condition from the alkaline extracts of the virus were only moderately successful, the notorious difficulties in separating protein and nucleic acid being accentuated by the small amounts of material available. In the most successful attempt the alkaline extract was $\frac{1}{8}$ saturated with ammonium sulphate, the protein precipitate was removed and the nucleic acid precipitated from the clear supernatant by acidification. The analysis of various preparations weighing from 1 to 3

mg. are given in Table VII ; the highest content of P found was 6·1 per cent. (theoretical 9·9 per cent.).

TABLE VII.—*Analyses of Nucleic Acid Fractions.*

No.	Per cent. P.	Per cent. thymonucleic acid calculated from		
		P content.	Orcin-HCl test.	Feulgen test.
1	3·1	31	28	++
2	4·6	46	53	40
3	6·2	61	60	—
4	3·2	32	37	++
5	4·7	46	49	—

For the Feulgen reaction an aliquot sample of the solution was heated with 0·1 volumes $N/2$ H_2SO_4 at 100° for 5 minutes, cooled, neutralized with $N/2$ NaOH and treated with 3 ml. Schiff's reagent, a control sample being tested without hydrolysis. By comparison of the colour developed after standing 10 minutes with that from known amounts of thymonucleic acid, rough estimates of nucleic acid were possible. Though the identification as nucleic acid is by no means rigorous, the correlation between the P content and the orcinol-HCl colour test, together with the Feulgen test, is sufficient to warrant the assumption that the fractions contain thymonucleic acid ; the presence of ribonucleic acid has not been excluded.

DISCUSSION.

The physical and chemical properties observed have been very constant in successive preparations of the virus.

Both the native and ether-extracted virus preparations show some boundary spreading in the centrifuge corresponding to a difference of about 15 per cent. between the sedimentation rates of the smallest and largest particles present. A difference of 15 per cent. in particle weights corresponds to one of only some $2\frac{1}{2}$ per cent. in particle diameters. Moreover, some of the boundary blurring may originate in endosmotic disturbances in the centrifuge cell (McFarlane, 1939). Clearly therefore a surprising degree of size homogeneity exists among the elementary bodies, provided one is justified in assuming that all the particles have the same density. The fact that the sedimentation rates of the native and ether-extracted virus are not detectably different indicates that the density of the ether-soluble material must be approximately one, and that size variations may indeed occur which do not affect the boundary homogeneity because of compensatory variations in mean particle density. If the ether-extractable material is acquired by the virus accidentally, for instance in the course of the violent emulsification of the skin pulp, one would expect it to have a random distribution among the virus particles, giving rise to particles of different lipin content. Such an heterogeneous population is nevertheless consistent with the degree of boundary homogeneity revealed by the centrifuge.

The adventitious lipoid material is probably held on the surface of the virus in the manner in which Langmuir conceives the formation of monolayers of fatty acids to take place, viz. more by virtue of the intense repulsion of the aqueous phase for hydrocarbon chains than by virtue of any specific attraction of the virus for the lipoid material. The diameter of the virus particle is approximately 200 mμ. A shell of lipin equivalent in weight to 10 per cent. of the whole (or approximately 13 per cent. by volume) can contain not more than 2 or 3 closely packed superimposed monolayers of oriented lipin (about 50 Å), but if the chains are not closely packed in each layer the thickness of the lipoid envelope will be greater and piling of layers must occur. It is probable indeed that the lipin molecules are not tightly packed, since the specific precipitation reactions shown by suspensions of the native virus demand a certain degree of penetrability of the envelope unless these reactions can be attributed to adsorbed specifically-precipitable material. The apparent membrane round the virus seen in ultra-violet photomicrographs (Barnard, 1935) is probably due to the presence of this highly refracting lipoid layer.

According to Smadel *et al.* (1938) and Elford (1938) the virus particle exhibits osmotic swelling in hypertonic media. The evidence for this is of an indirect nature based on measurements of sedimentation rates in sucrose solutions of different concentration and is open to certain objections. The most important of these objections are that, on the evidence of Smadel *et al.*, the sedimentation rate of the particle is not the same in iso-osmotic media of sucrose, glycerol and urea ; and that the viscosity corrections which they use are seriously inexact since they ignore electro-viscous effects on sedimentation rate. The results which we have given of measurements of particle hydration by a more direct method indicate that osmotic considerations can play only an insignificant part in determining the total amount of water associated with the virus. This is shown for example by the fact that the hydration is almost the same in 0·74 per cent. sucrose and 1·52 per cent. egg albumin in spite of the enormous difference in the osmotic pressures of the two. The values obtained for the hydration are so high that they preclude the possibility that any great part of this water is held within a membrane. If this were the case the density of the particle would be very little more than that of the aqueous medium in which it is suspended, and the rapid sedimentation rate would correspond to a size of particle large enough to be visible in the ordinary microscope. Most of the 7–8 vols. of water associated with the virus substance clearly cannot sediment with the virus and must be visualized as surrounding the particle in the form of an ion atmosphere. The presence of this atmosphere tells us nothing about the state of hydration of the central particle.

The extraordinary resistance of the virus particle to attempts at disruption has been noted by various workers. Mechanical grinding of a violent nature or repeated freezing and thawing produces no obvious disruption of the particles. The virus may be taken from aqueous suspension directly into saturated sucrose and back again into water without any evidence of cell disruption or loss of infectivity (Smadel *et al.*, 1938), and it is not affected by urea in concentrations which hæmolyse red blood-cells.

The basis of many animal cell membranes is believed to be a lipo-protein duplex film (*cf.* Harvey and Danielli, 1938) which is notoriously sensitive to drying and to lipoid solvents. We do not know of any animal cell with a semi-permeable membrane which is unaffected by drying and extraction with ether and benzene in the manner described here for the elementary body. It is known that certain bacteria, *e. g.* pneumococci (Gay *et al.*, 1931), can withstand such treatment, but it is not considered that a *prima facie* case has been made out for the existence of a semi-permeable membrane on the surface of such bacteria. It appears to us that the weight of evidence is against the conception that the virus particle is limited by a semi-permeable membrane functioning on osmotic principles, and that it is more in keeping with the properties of the particle to postulate a rigid or semi-rigid structure akin to a giant protein molecule.

In trying to visualize the architecture of such a structure several alternatives present themselves. An attractive hypothesis is that the virus particle is built up of " bricks " of specifically-precipitable protein cemented together by the nucleic acid and lipoid material. The course of the disintegration in sodium carbonate solution is not inconsistent with such an idea, since particles ranging in size from those little smaller than the original to others small enough to pass through a cellophane dialyser, but not apparently very different chemically, were produced. It is a moot point whether this disintegration was caused by the rapid removal of the nucleic acid. It is possible that if the nucleic acid and lipin could be removed by agents which have no effect on the protein a disaggregation into homogeneous protein units might follow.

Short-range chemical linkages are believed to hold together the constituent parts of a plant virus molecule ; the difficulty in accepting a similar type of structure in the case of vaccinia virus is perhaps mainly subjective, since it is so much greater in size and more complex in chemical constitution than anything hitherto accepted as a molecule. The alternative idea that we are concerned with a protein gel maintained by long-range physical attractions is for that reason more acceptable, especially since it suggests some relationship between the virus and the gel-like cytoplasm of animal cells, which is of comparable chemical complexity. This view would rest on a firm basis if unequivocal evidence could be adduced for the presence of a considerable quantity of water *inside* the virus particle. There is, however, no such evidence ; on the contrary the intense light-scattering which the particles display by dark-ground illumination suggests that they have a high density and a small degree of hydration.

It appears therefore that while this virus is more complex chemically than the plant viruses, there are no grounds for believing that its structural integrity depends on any fundamentally different physical principle.

SUMMARY.

The degree of hydration of the particle in homogeneous suspensions of the elementary bodies of vaccinia virus has been measured by a partition

method ; the particle has large amounts of water associated with it in the form of an atmosphere which is not penetrated by sucrose and egg albumin.

Elementary bodies can be dried, extracted with ether or benzene and resuspended in an aqueous medium to the same degree of dispersity as the original homogeneous suspension without detectable loss in infectivity. The physical and chemical properties of the residual particle, which must be considered to be the virus, are described.

The virus contains lipoid material which can only be removed by alcohol, as well as protein, carbohydrate and nucleic acid. Its behaviour to mechanical, osmotic and chemical agents suggests that it has no superficial membrane, and the possible structural forces which could maintain the integrity of the particle are discussed.

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ALUM-PRECIPITATED DIPHTHERIA TOXOID.

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It has been known for many years that bacterial toxins can be precipitated from culture filtrates by the addition of metallic salts, and, as early as 1889, Roux and Yersin used potash alum in the attempt to purify diphtheria toxin, but it was not until Glenny, Pope, Waddington and Wallace (1926) showed that the precipitate obtained when diphtheria toxoid and alum were mixed possessed increased antigenic efficiency as compared with the untreated toxoid, that the importance of alum-precipitated toxoid was fully appreciated. In 1931 Glenny and Barr worked out in detail the conditions necessary for the precipitation of diphtheria toxoid by alum, and showed that the purity of the precipitates expressed in Lf units per mg. of nitrogen increases considerably as the amount of added alum is increased, but the quantity of aluminium detected in the precipitates decreases. Smith (1932) investigated the effect of adding varying quantities of alum to toxoid followed by adjustment of the mixtures with acid or alkali to arbitrary pH values. She found that, for a given quantity of alum, the percentage of toxoid precipitated at pH 4·8 was greater than at pH 7·0, and that the purity obtained was slightly greater at the lower pH. Ando, Komiyama and Manako (1936), using toxoid from a semi-synthetic medium, found that for a given amount of alum the percentage of toxoid precipitated varied with the pH to which the mixture was adjusted. They found also that with partially purified toxoids soda has to be added to the alum toxoid mixture to bring the pH to the optimum for removal of toxoid from solution. Using purified toxoid they obtained alum precipitates of a purity of about 200 Lf units per mg. of nitrogen.

For human immunization against diphtheria the purity of the preparation may be of great importance. The purer the product, the less expectation is there of non-specific reactions occurring, and probably more specific antigen could be injected without causing undue disturbance. Work has therefore been carried out to try and secure products of exceptionally high purity so that these points may be investigated clinically. During the past few years the work of Eaton (1936) and Pappenheimer (1937) has resulted in the isolation of diphtheria toxin in what would appear to be a pure state. It has the properties of protein, is readily denatured by acid and heat coagulable. These workers found that the isolated toxic protein contained 0·00046 mg. of nitrogen per Lf unit. If it is assumed that the conversion of toxin into toxoid by formaldehyde does not involve any change in the nitrogen content, then the

value of 0·00046 mg. nitrogen per Lf unit, of 2146 Lf units per mg. nitrogen may be taken as a basis for purity determinations for toxoid. If the papers of the workers mentioned earlier are studied, it will be seen that the alum precipitates of crude toxoids contain a considerable quantity of non-specific nitrogen, for the purities obtained fall far short of the value which one would expect from Pappenheimer and Eaton's work. At the commencement of this work, Dr. C. G. Pope suggested that washing of the alum precipitates with ½ per cent. disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) might result in removal of some of the non-specific nitrogen for a very small loss of Lf units, and this procedure has proved of such great value that it has been used extensively in the experiments to be recorded.

EXPERIMENTAL.

The required volume of alum was added in the form of a 10 per cent. solution to the toxoid and the mixture allowed to stand overnight at room temperature, since Glenny and Barr (1931) have shown that precipitation is not complete until 18 hours, at least, have elapsed. The resulting precipitate was centrifuged down and the pH of the supernatant determined (glass electrode). The Lf value of the supernatant was then found by blending with a fast flocculating toxin of known titre (Glenny, Pope, Waddington and Wallace, 1926) after the addition of 5 per cent. of solid sodium citrate (Smith, 1932) to prevent precipitation of the blend toxin by excess of alum in the supernatant.

The precipitate was washed twice with 1 per cent. saline, and then eluted overnight with a volume of ½ per cent. disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) equal to the volume of toxoid used for the preparation of the precipitate. Next day the eluted precipitate was again centrifuged down, the Lf value and total nitrogen content of the eluate determined, and the precipitate emulsified in saline to a volume equal to, or occasionally one-half of, the volume of toxoid used for the preparation of the precipitate.

The Lf value of the emulsion was determined by bringing a part into solution by the addition of 5 per cent. of solid sodium citrate, while aluminium estimations, where done, were carried out using the method of Pope (1931). All nitrogen estimations were done using the micro-Kjeldahl technique, and employing selenium oxychloride to ensure complete oxidation of organic matter.

Results Obtained with Toxoids made from Broths of High Nitrogen Content.

In 1932 Pope and Smith (see also Pope and Healey, 1933) published details of the method of preparation of broths which would give regularly toxoids of 50–70 Lf units per c.c. These broths were made by a 6-hour tryptic digest of beef muscle and had a total nitrogen content of approximately 6 mg. per c.c. They gave with the strain of *C. diphtheriæ* in use at these Laboratories a very luxurious surface film with heavy " curtains " and formation of large deposits during growth. The results obtained by the addition of varying quantities of alum to a typical toxoid made from this type of broth are shown

TABLE I.—*Showing the Purities Obtained by Precipitation of Toxoid of High Nitrogen Content with Alum (½ per cent. Phosphate Elution).*

Toxoid: TMX 939. Lf = 68 units per c.c. Total nitrogen = 5.5 mg. per c.c.

10 per cent. alum per 100 c.c.	Supernatant.		½ per cent. phosphate eluate.			Final emulsion of washed precipitate.						
	pH.	Lf units per c.c.	Lf units per c.c.	Total nitrogen mg./c.c.	Lf units per mg. N.	Lf units per c.c.	Total nitrogen mg./c.c.	Lf units per mg. N.	Aluminium mg./c.c.	Lf units per mg. aluminium.	Aluminium precipitated. (per cent.)	Yield of toxoid precipitated. (per cent.)
10 c.c.	6·93	62	1·5	0·010	150	1·5	..	..	0·063	23·8	11·0	2·2
20 ,,	6·29	31	16	0·218	73	18	0·189	95	0·380	47	33·4	26·4
30 ,,	5·58	3	9	0·353	25	53	0·463	114	1·18	45	69·5	78·0
40 ,,	4·96	2	2	0·331	6	63	0·512	123	1·18	53·4	51·6	92·5
50 ,,	4·62	2	2	0·278	7	62	0·480	129	1·17	53	41·1	91·0
60 ,,	4·44	3	1·5	0·209	7	56	0·378	148	1·11	50·5	32·5	82·4
70 ,,	4·34	2·5	7·5	0·136	55	51	0·256	192	0·575	88·5	14·4	75·0

in Table I. The results shown are representative of a large number of this type of toxoid which were investigated. In each case the washed alum precipitate was eluted with a volume of ½ per cent. disodium hydrogen phosphate equal to the volume of toxoid used for the preparation of the precipitate.

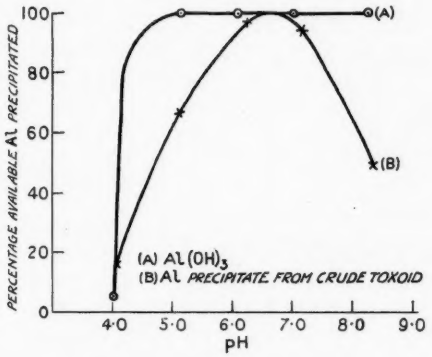
It will be seen that—

(*a*) Almost complete precipitation of toxoid occurs at pH values below 5·5.

(*b*) Maximal precipitation of the available aluminium occurs over a pH range of pH 4·5–5·5, but never even approaches 100 per cent.

(*c*) The purity of the product, both in terms of Lf units/mg. nitrogen and Lf units/mg. Al, increases with fall of pH.

(*d*) The ½ per cent. phosphate elution removes an appreciable quantity of the non-specific nitrogen for a very small loss in Lf units.



Nevertheless, the results are somewhat disappointing when it is remembered that a purity of 2146 Lf units/mg. nitrogen would be attained if the precipitate were free from non-specific nitrogen, and it was concluded that toxoids made from such broths of high nitrogen content were unsuitable material for the preparation of alum precipitates of high purity.

Results Obtained with Toxoids made from a Special Broth of Low Nitrogen Content.

Recently Pope and Linggood (1939) have devised a broth of much lower nitrogen content (2·0–2·4 mg./c.c.), on which the organism grows throughout the usual 10-day incubation period almost entirely as a surface film, and does not form the heavy deposits so characteristic of the growth of *C. diphtheriæ* on broths of high nitrogen content. The Lf values produced from this special broth are usually between 30 and 40 Lf units/c.c., and the ratio of theoretical heat-coagulable protein (as calculated from Pappenheimer's figures) to the heat-coagulable protein actually found by experiment in the culture filtrates from this new type of broth is usually higher than the corresponding figure for toxins made from other types of broths. With toxoids made from this new

TABLE II.—*Showing the Purities Obtained by Precipitation with Alum of a Toxoid made from a Special Broth of Low Nitrogen Content, Before and After Charcoal Treatment.*

Toxoid: TMZ 1612. Lf = 39·5 units per c.c. Total nitrogen = 2·19 mg. per c.c. pH 7·8.

Analyses of starting material.					10 per cent. alum per 100 c.c.	No phosphate treatment.				With phosphate treatment.				
						Supernatant.		Final emulsion.		Supernatant.			Final emulsion.	
Per cent. charcoal.	Lf units per c.c.	Total nitrogen mg./c.c.	Purity Lf/mg. N.	Ratio.		pH.	Lf units per c.c.	Purity Lf/mg. N.	Yield (per cent.†)	pH.	Lf units per c.c.	Phosphate eluate Lf/mg. N.	Purity Lf/mg. N.	Yield (per cent.†)
No charcoal	39·5	2·19	19·7	40%	4 c.c.	6·84	Discarded; precipitate too small.							
					6 ,,	6·50	25	117	42	6·57	19·5	134	155	21·5
					10 ,,	5·82	4·5	143	90	5·85	4·5	126	242	25·0
					14 ,,	5·38	1·5	138	92·5	5·40	1·5	38	259	82
					20 ,,	5·01	0	148	82	5·02	0	17	265	92·5
					30 ,,	4·60	0	190	87	4·64	1·5	32	276	87
					40 ,,	4·36	7*	375	80	4·40	1·5	142	345	62
3	32·5	1·23	26·4	50%	4 c.c.	6·53	Discarded; precipitate too small.							
					6 ,,	6·20	10·5	202	75	6·21	10·5	169	256	32
					10 ,,	5·55	1·5	210	97	5·55	1·5	138	438	57
					14 ,,	5·16	1·5	214	97	5·18	0	83	481	78·5
					20 ,,	4·84	0	235	97	4·88	0	19	425	94
					30 ,,	4·49	3·5*	364	91	4·53	0	64	440	81·5
					40 ,,	4·27	5*	511	78	4·31	3·5	Solution complete in phosphate.		
5	28·5	0·876	32·6	67%	4 c.c.	6·70	16·5	258	47	6·72	15·5	286	329	38
					6 ,,	6·25	5·5	264	86	6·30	5·5	218	430	51
					10 ,,	5·60	1·5	244	100	5·61	1·5	118	520	75
					14 ,,	5·20	0	246	100	5·20	0	90	526	75
					20 ,,	4·85	0	266	96	4·84	1·5	30	535	100
					30 ,,	4·54	0	362	100	4·47	0	139	704	82·5
7	19·5	0·642	30·4	89%	4 c.c.	6·78	6·5	496	68	6·85	8·5	485	651	29·5
					6 ,,	6·25	1·5	445	95	6·35	1·5	333	698	52·6
					10 ,,	5·56	1·5	382	90	5·60	1·5	149	766	76
					14 ,,	5·20	0	358	90	5·22	0	124	747	81
					20 ,,	4·86	1·5	531	94	4·89	0	69	700	86
					30 ,,	4·51	1·5	564	99	4·53	0	195	813	76

* Some secondary precipitation after spinning. Therefore purities somewhat high.
† Excluding charcoal treatment.

medium, it was found that the " ratio " was considerably improved by treatment with charcoal.* It was therefore considered to be of interest to investigate the effect of alum on toxoids made from this medium both before and after charcoal treatment. As is to be expected, such toxoids are much more readily precipitable with alum. The purities obtained are briefly summarized in Table II.

From this table it will be seen that the purities obtained are higher than those secured with the older type of toxoid, and the beneficial effect of the elution with ½ per cent. phosphate and of charcoal treatment prior to precipitation is clearly demonstrated. Although treatment with 7 per cent. charcoal results in a loss of 50 per cent. of the specific toxoid in the starting material, the final purities of the alum precipitates are three to four times higher than those obtained from the untreated toxoid. It should be noted that, although the initial purity of the charcoal filtrates as expressed by the Lf units per mg. nitrogen figure reaches a maximum with this particular toxoid with 5 per cent. charcoal, the purities of the resulting alum precipitates do not become maximal with this amount of charcoal, but appear to increase rather as the " ratio " of the toxoid is improved. This fact seems to indicate that with crude toxoids part of the non-specific nitrogen present in the resulting alum precipitate is due to the heat-coagulable protein, other than the toxin protein, present in the toxoid. Another point of interest, which will be referred to later, is the fact that with increasing amounts of charcoal the resulting filtrates show more complete precipitation of toxoid at higher pH values; for example, with no charcoal treatment, the toxoid gave no appreciable precipitate with 4 c.c. of 10 per cent. alum per 100 c.c., while after treatment with 7 per cent. charcoal definite precipitation occurred, and only 6·5 Lf units/c.c. were left in the supernatant, although the pH of the mixture was almost identical in both cases.

Results Obtained with Ultrafiltered and Washed Toxoids.

Ultrafiltration has been used extensively by Wadsworth and Quigley (1934) as a means of purifying toxoids, and recently Pope and Linggood (1939) have shown that charcoal treatment of toxoids made from suitable broths of low nitrogen content can give purities up to 1200 Lf units per mg. nitrogen when followed by ultrafiltration.

It was therefore considered to be of interest to investigate the addition of alum to such purified toxoids. It was found that even the addition of small amounts of alum caused a drop in the pH of the mixture to 4·0 or below, with the result that only a very small flocculent precipitate was formed, probably due merely to acid precipitation of the toxoid. It was found, however, that the addition of soda would produce a bulky precipitate, and if the pH was measured for each additional amount of soda added, a precipitation curve could be obtained similar in shape and position to that produced when an aluminium salt is titrated with soda. Table III shows the results obtained when alum is added concurrently with soda to an ultrafiltered and washed toxoid so as to give a range of pH values.

* The brand known as " Eponit " was used.

TABLE III.—*Showing the Effect of the Addition of Alum and Soda Concurrently to an Ultrafiltered and Washed Toxoid.*

Mixtures of 100 c.c. purified toxoid RX 3230 and 10 c.c. of 10 per cent. alum. Lf. of RX 3230 = 32·5 units per c.c.

No.	N/1 NaOH (c.c.).	pH.	Lf units in filtrate.	Lf units in precipitate.*
A	0·25	3·92	4	..
B	2·0	4·35	0	..
C	2·3	4·85	0	..
D	2·4	5·26	0	..
F	2·5	5·93	0	32·5
H	2·6	6·34	0	33·5
K	2·7	6·90	1	33·0
L	2·8	7·78	2	30·5
N	3·5	9·50	5	..

* Precipitate dissolved in 5 per cent. citrate.

It will be seen from Table III that toxoid is precipitated quantitatively from solution over a considerable range of pH values, and even at pH 7·8 only 2 units are left in the supernatant, a pH value at which crude toxoids give no precipitate with alum. It will also be noted that the addition of soda is very critical around pH 6·0–7·5. Hence an attempt was made to add the necessary base as sodium bicarbonate prior to the addition of alum. 1 per cent. $NaHCO_3$ was found to be the most suitable concentration. This amount only raised the initial pH of the toxoid from pH 7·4 to pH 8·1–8·3, and after the addition of one-tenth volume of 10 per cent. alum the resulting pH was 6·6–7·0. Using highly purified toxoids (1200 Lf units/mg. N) diluted out to an Lf value of 100 units/c.c., it was found that such a procedure removed 97 per cent. of the toxoid from solution and yielded alum precipitates of purities 1300–1400 Lf units/mg. of nitrogen.

This value is a great improvement on the purities obtained even from the precipitation of charcoal-treated toxoids. It appears, therefore, if high purity preparations of alum-precipitated toxoid are required, it would seem necessary to purify the toxoid extensively before precipitation with alum, using preferably a method of purification that does not involve drastic chemical treatment which would injure the antigenic nature of the specific toxoid. This can be done most readily on a large scale by using a broth of low nitrogen content and then treating the resulting toxoid heavily with charcoal. The charcoal filtrate can then be further purified by ultrafiltration or adsorption and elution from Willstätter cream, the product in the latter case being dialysed free from phosphate. Such a procedure should enable much purer preparations of alum-precipitated toxoid to be made.

The Nature of the Formation of A.P.T.

Crude toxoids contain a variety of nitrogenous materials, amino acids, polypeptides, proteoses and, as Pope and Linggood have recently shown,

heat-coagulable proteins other than the toxin protein. It is therefore difficult to postulate what happens when alum is added to such a complex mixture, but it is evident from the foregoing results that the process does not merely involve the precipitation of aluminium hydroxide and the adsorption of the toxoid thereon. Aluminium hydroxide is quantitatively precipitated from solution of its salts over a pH range 4·2–8·0, and yet little or no precipitate is formed when alum is added to crude toxoids until a pH 6·5 is reached, although sufficient alum has been added to produce an appreciable amount of precipitate if the aluminium had been precipitated quantitatively as $Al(OH)_3$. From Table I it will be seen, however, that the maximum percentage of the available aluminium precipitated occurs at pH 5·5, but even at this value, does not reach 100 per cent.

In order to demonstrate these points more clearly, 100 c.c. portions of a crude toxoid were taken and to each 30 c.c. of 10 per cent. alum were added. The pH of the resulting mixture was then 5·0. The mixtures were then adjusted with normal acid or alkali to give pH values of 4, 5, 6, 7 and 8. Some difficulty was experienced in adjusting the pH values on the alkaline side of the pH. 5·0; the pH values were unsteady and continued to fall, but each mixture was repeatedly adjusted until the pH became constant at the required value. Similarly, mixtures of 30 c.c. of 10 per cent. alum and 100 c.c. of water were adjusted to a series of pH values, but, although the buffering effect of amino acids, etc., was lacking, with these mixtures there was little sign of instability even at pH 8·0. Finally each mixture of both series was made up with water to a constant volume (150 c.c.). 50 c.c. amounts of each mixture were then centrifuged and the precipitate washed twice with water and finally emulsified to 50 c.c. The aluminium content was then determined. It should be noted that the precipitate from the pH 4, 5 and 6 mixtures of the toxoid and alum were readily soluble in borax; the pH 7 and 8 mixtures were dissolved with less ease. All the control mixtures were difficult to dissolve in borax. The results of this experiment are shown graphically in Curves A and B. As was to be expected, with the control mixtures of alum and water the aluminium was precipitated quantitatively over a pH range 4·2–8·0. In the presence of toxoid, however, the aluminium is only quantitatively precipitated at about pH 6·5 and on either side of this optimum decreases rapidly. This optimal pH value for the precipitation of the aluminium may, at first sight, appear to be in contradiction to the results obtained in Table I, where it will be seen that the maximal precipitation of the available aluminium occurs over a pH range 4·5–5·5. It will be appreciated, however, that there is a distinct difference between the conditions of the two experiments. In the former case, sufficient alum was added to the toxoid to lower the pH of the mixture to pH 5·0, and then a certain amount of soda added to bring the pH up to the optimum. Thus the quantity of base available for the precipitation of the aluminium is totally different from the experiment described in Table I, where the only base available is that due to the natural alkalinity of the toxoid, which will itself vary with different toxoids.

Thus it is evident that the process involves the formation of complexes between the aluminium and the proteins and their degradation products present in the crude toxoid. Many of these complexes are soluble over a

considerable part of the pH range 4·2–8·0 and therefore prevent the quantitative precipitation of the aluminium as $Al(OH)_3$. The reason for the increased purity of alum precipitates as the pH is lowered by the further addition of alum is therefore apparent, for the solubility of the aluminium protein complex increases as one descends the pH scale. When, however, a crude toxoid is treated with charcoal the latter removes some of these proteins, proteoses, etc., and then, as can be seen from Table II, the pH range over which precipitation occurs, and therefore over which removal of toxoid from solution takes place, becomes greater. When ultrafiltered and washed toxoids are used it will be seen that precipitation occurs throughout the pH range 4·2–8·0 and subsequent removal of toxoid is practically complete since, it is thought, the nitrogen-containing substances with which alum can form complexes have been reduced to a minimum. In such cases there is every reason to suppose that the toxoid is removed principally by adsorption on to the $Al(OH)_3$ precipitated *in situ*, whereas in the present state of our knowledge any definite statement as to whether the toxoid protein itself is removed from crude toxoids by adsorption or by precipitation as an aluminium salt must be a matter of conjecture.

The purities obtained by alum precipitation of charcoal-treated toxoids made from low nitrogen broths are considerably less than the purities which can be obtained from such toxoids when purified by other methods. For example, TMZ 1612 when treated with 7 per cent. charcoal and the filtrate adsorbed on to Willstätter cream at pH 6·0, gave an eluate with 2 per cent. $Na_2HPO_4.12H_2O$ whose purity was 1200 Lf units per mg. nitrogen, a purity far above that obtained by alum precipitation even when the precipitate has been eluted with ½ per cent. phosphate. Possibly this may be due to the fact that the formation of complexes between the aluminium and the nitrogenous impurities in the toxoid occurs more readily when the alum is added to the toxoid and the precipitate formed *in situ*, than when the toxoid is treated with the relatively inert Willstätter cream. The fact that the addition of alum to toxoid removes polypeptides, etc., from solution was demonstrated by the following experiment: Toxoid TMZ 1612 was treated with 7 per cent. charcoal and the filtrate ultrafiltered through 8 per cent. parlodion membranes which retained the toxoid quantitatively. The ultrafiltrate, which gave no precipitate even when saturated with ammonium sulphate, was treated with 280 c.c. per litre of 10 per cent. alum. The resulting precipitate was washed twice with saline in the routine manner, and on emulsifying to one-tenth of the original volume was found to contain nitrogen (0·183 mg. per c.c.). It was found possible to elute most of this nitrogen by the addition of $Na_2HPO_4.12H_2O$ to 5 per cent. Both the total nitrogen and the amino nitrogen* in this eluate were then estimated and found to be 0·136 mg. per c.c. and 0·024 mg. per c.c. respectively, giving an amino nitrogen to total nitrogen ratio of 17·4 per cent. Some of the eluate was then hydrolysed in a sealed tube for 24 hours with hydrochloric acid (final concentration 20 per cent. hydrochloric acid). After neutralization the amino nitrogen to total nitrogen ratio was again determined and now found to be 83 per cent. Hence

* The amino nitrogen was determined by a micro-adaptation of the method of Pope and Stevens (1939).

it can be concluded that the nitrogen present in the alum precipitate formed from the ultrafiltrate was a polypeptide, which is not heat-coagulable or precipitable by ammonium sulphate. If, therefore, the addition of alum to the ultrafiltrate from a charcoal-treated toxoid can result in the formation of a precipitate containing nitrogen, the obvious inference is that the alum precipitate from the toxoid itself will contain much more of the non-specific polypeptide and proteose nitrogen, etc. Hence the comparatively low purities obtained by the alum precipitation of crude toxoids.

SUMMARY.

1. The addition of alum in varying amounts to diphtheria toxoids made from broths of high and low nitrogen content has been investigated with particular regard to the purities of the resulting precipitates.

2. Toxoids of low nitrogen content (2·0–2·4 mg./c.c.) give alum precipitates of higher purity than toxoids having a higher nitrogen content (5·0–6·0 mg./c.c.), this being especially the case when the precipitate is eluted with $\frac{1}{2}$ per cent. $Na_2HPO_4.12H_2O$.

3. Treatment of toxoids of low nitrogen content with charcoal prior to alum precipitation results in a considerable increase in the purity of the alum precipitates, and precipitates of a purity of 700–800 Lf units/mg. nitrogen have been made by this method.

4. Precipitates of even higher purity can be made if the toxoid is purified more extensively before precipitation. After charcoal treatment and ultrafiltration of toxoids made from broths of low nitrogen content, alum precipitates of a purity of 1300–1400 Lf units/mg. nitrogen have been produced, providing a base is added in a convenient form such as sodium bicarbonate prior to precipitation.

5. The nature of the formation of alum-precipitated toxoid is discussed.

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FURTHER STUDIES ON THE ABSORPTION, MOBILIZATION AND EXCRETION OF LEAD.

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THE ABSORPTION OF LEAD.

In previous communications the methods of analysis and the composition of the animal diets used have been described (Tompsett, 1939*a*, *b*), and it has been shown that lead is absorbed readily on a low calcium diet but with difficulty on a high calcium diet and suggestions were put forward to account for this (Tompsett, 1939*b*). Telfer (1939) has shown that the addition of hydrochloric acid to the diet increased the amount of calcium and magnesium absorbed from the alimentary tract. In view of this it was considered that acidification of the diet would aid the absorption of lead.

Adult male mice were placed on a high calcium diet containing lead acetate at the rate of 1 mg. Pb per mouse per day, and 0·5 ml. of N hydrochloric acid per mouse per day for a period of 14 days. At the end of this period the animals were placed upon the high calcium diet alone for 4 days to remove unabsorbed lead from their alimentary tracts and killed. Their total lead content was estimated and the results are shown in Table I.

TABLE I.—*Adult Male Mice. High Calcium Diet + 1 mg. Pb (lead acetate) + 0·5 ml. N HCl per mouse per day. Period, 14 days.*

	Weight. (g.)	Total lead. (mg.)	Lead. (mg. Pb per 100 g.)
1	28	1·429	5·10
2	19	0·909	4·78
3	21	1·111	5·29
4	21	1·176	5·60
5	23	1·000	4·35
6	17·5	0·667	3·81
7	21·5	0·909	4·23
8	31·5	1·111	3·53
		Average	4·58

It was previously found that mice receiving high and low calcium diet and lead acetate at the rate of 1 mg. per mouse per day for a period of 14 days, contained on an average 0·215 and 2·63 mg. Pb per 100-g. mouse respectively

(Tompsett, 1939*b*). In the experiment described in this paper the mice contained on an average 4·58 mg. Pb per 100-g. mouse. These results show that the addition of hydrochloric acid to the diet favours the absorption of lead from the alimentary tract.

THE MOBILIZATION OF LEAD.

In a previous communication the effect of certain agents on the mobilization of lead was examined (Tompsett and Chalmers, 1939). In men with histories of abnormal exposure to lead it was found that low calcium intake, with or without ammonium chloride, produced a rise in blood lead. High calcium intake was found to produce a fall in blood lead. In mice a high calcium intake resulted in lead being transferred from the soft tissues to the skeleton, whereas a low calcium intake had the reverse effect.

To produce mobilization of lead in man the following are the commonest substances used : ammonium chloride, potassium iodide, sodium bicarbonate. It is usual to use ammonium chloride in conjunction with a low calcium diet, and the other two in conjunction with what is termed an ordinary diet.

Adult male mice were placed on low calcium diet containing lead acetate at the rate of 1 mg. Pb per mouse per day for a period of 14 days. They were placed on high calcium diet for 7 days to produce a transference of lead from the soft tissues to the skeleton. They were then placed in groups on various diets for 7 days and killed. The lead content of their soft tissues and skeletons was estimated separately. The percentage of the total lead present in the soft tissues and skeleton was then calculated. The results are shown in Table II. It will be seen that ammonium chloride, potassium iodide and sodium bicarbonate produced mobilization of lead on both high and low calcium intake.

THE EXCRETION OF LEAD.

Kehoe *et al.* (1933) have shown that, in man, the quantity of lead excreted decreases rapidly after removal from exposure until it does not greatly exceed the normal, at which level it remains for a long period. It was considered of interest to estimate the quantity of absorbed lead that could be excreted during a period after removal from exposure.

Adult male mice were placed upon low calcium diet containing lead acetate at the rate of 1 mg. Pb per mouse per day for a period of 14 days. They were then placed upon high calcium diet for 7 days to remove unabsorbed lead from their alimentary tracts and to produce a transference of lead from soft tissues to skeleton. The animals were then placed in separate glass jars floored with several layers of filter-paper for 14 days. Half of their number were placed on a high calcium diet and the remainder on low calcium diet. The animals were then killed and their total lead content was estimated. The lead content of the contents of the jars was also estimated. The quantity of lead excreted during the period was then calculated after making allowances for the lead content of the filter-paper and diet consumed during the period. The percentage of the total original lead excreted was then calculated. The results,

TABLE II.—*Mobilization of Lead by Addition of 50 mg. per Mouse per Day of Ammonium Chloride, Potassium Iodide and Sodium Bicarbonate to Low and High Calcium Diets.*

Low Calcium Diet.

	No addition.			+ ammonium chloride.			+ potassium iodide.			+ sodium bicarbonate.		
Mouse No.	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
Lead content of soft tissues (mg.)	0·050	0·050	0·066	0·110	0·136	0·104	0·091	0·146	0·121	0·110	0·100	0·108
Lead content of skeleton (mg.)	0·100	0·091	0·132	0·140	0·148	0·110	0·099	0·154	0·146	0·800	0·140	0·140
Total lead (mg.)	0·150	0·141	0·198	0·250	0·284	0·214	0·190	0·300	0·267	0·190	0·240	0·248
Per cent. lead in soft tissues	33·4	35·5	33·6	44	48	48·6	48	48·7	45·3	57·9	41·6	44
Per cent. lead in skeleton	66·6	64·5	66·4	56	52	51·4	52	51·3	54·7	42·1	58·4	56

High Calcium Diet.

	No addition.			+ ammonium chloride.			+ potassium iodide.			+ sodium bicarbonate.		
Mouse No.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.
Lead content of soft tissues (mg.)	0·049	0·039	0·066	0·124	0·081	0·104	0·082	0·090	0·100	0·080	0·090	0·104
Lead content of skeleton (mg.)	0·144	0·145	0·233	0·125	0·098	0·106	0·077	0·040	0·120	0·160	0·090	0·111
Total lead (mg.)	0·193	0·184	0·299	0·249	0·179	0·210	0·159	0·130	0·220	0·240	0·180	0·215
Per cent. lead in soft tissues	25·4	26·7	23	50	45·2	49·5	51·6	69·2	45·4	33·3	50	48·3
Per cent. lead in skeleton	74·6	73·3	77	50	54·8	50·5	48·4	30·8	54·6	66·7	50	51·7

shown in Table III, indicate the rapidity with which recently absorbed lead may be eliminated. On low calcium intake, approximately two-thirds of the total lead was excreted in 14 days, and on high calcium intake approximately one-quarter of the total lead was excreted during the same period.

TABLE III.—*Lead Excreted in* 14 *days on Low and High Calcium Diets.*

Low Calcium Diet.

Mouse No.	1.	2.	3.	4.
Total lead (mg.)	0·105	0·150	0·141	0·099
Excreted lead (mg.)	0·175	0·225	0·220	0·198
Original total lead (mg.)	0·280	0·375	0·361	0·297
Per cent. original total lead excreted	62·5	60	60·9	66·7

High Calcium Diet.

Mouse No.	5.	6.	7.	8.
Total lead (mg.)	0·193	0·184	0·289	0·320
Excreted lead (mg.)	0·060	0·049	0·068	0·082
Original total lead (mg.)	0·253	0·231	0·357	0·402
Per cent. original total lead excreted	23·7	21·2	19	20·4

DISCUSSION.

It has been shown that the addition of hydrochloric acid to the diet increases the absorption of lead from the alimentary tract. Gastric acidity, which can show great variations, is an important factor in the absorption of lead.

Ammonium chloride, potassium iodide and sodium bicarbonate have been shown to effect mobilization of lead on both high and low calcium intake. This suggests that such substances as potassium iodide and sodium bicarbonate should not be administered to persons who have absorbed large amounts of lead unless mobilization of lead is desired, otherwise toxic symptoms are liable to develop. High calcium intake under such conditions would be ineffective. Common offenders in this respect are "stomach powders", the majority of which contain sodium bicarbonate. That many lead-workers admitted to the Glasgow Royal Infirmary with symptoms of lead-poisoning had been treated with such powders was shown by the presence of large amounts of bismuth in their fæces. The irresponsible use of "stomach powders" by lead-workers is a potential danger.

After removal from exposure to lead, absorbed lead may be eliminated very rapidly. It frequently happens that patients, with histories of abnormal exposure to lead, are not admitted for examination until some time has elapsed since the last exposure, and as a result the quantity of lead excreted may not greatly exceed the normal. It cannot be assumed that, under such conditions, an almost normal lead excretion suggests a mild exposure.

The question of susceptibility often arises in lead-poisoning. The writer considers that variation in diet, gastric acidity, administration of drugs, metabolic disorders, etc., play an important part.

In all the experiments in which mice were used, the administration of relatively large amounts of lead appeared to have little effect upon the general bodily health of the animals.

SUMMARY.

The addition of hydrochloric acid to the diet increases the absorption of lead from the alimentary tract.

Ammonium chloride, sodium bicarbonate and potassium iodide produce mobilization of lead on both a low and a high calcium intake.

Recently absorbed lead may be excreted very rapidly after removal from exposure.

The question of susceptibility is discussed.

I wish to thank Dr. A. B. Anderson for his helpful criticism and advice.

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SURVEY OF PAPERS.

AUGER describes a simple method of culturing sputum on solid media in the presence of extra moisture and a high concentration of carbon dioxide. In such conditions maximum growth of pneumococci is possible in 15 hours. The method also encourages the growth of other respiratory pathogens (p. 439).

Baker and McIntosh showed in 1927 that trypsin increased the infectivity of suspensions of the Rous Sarcoma No. 1. SELBIE AND MCINTOSH now find that trypsin has a similar action on filterable and most non-filterable chemically-induced fowl tumours. Peptic and autolytic digestion, and repeated freezing and thawing have a similar action, produced apparently by the breaking up of tissue fragments and the release of virus particles. The transmission of fowl tumours by cell-free filtrates seems to depend to a great extent on the virulence of the tumour, and it is possible, by the adoption of suitable methods, to increase the virulence of some apparently non-filterable tumours so that they become filterable (p. 443).

MACLEAN, SHEPPARD AND MCHENRY have examined the tissue changes resulting from acute deficiency of ascorbic acid in guinea-pigs. There is a failure in those specialized cells, odontoblasts, osteoblasts, etc., which are concerned in the formation of calcific tissue, and the changes are confined to the bones and teeth. There is no evidence to support the view that the changes are the result of the failure of specialized cells to secrete some substance which normally produces jellying of collagenous material. The inanition coincident with the development of ascorbic acid deficiency is not responsible for any of the observed histological changes (p. 451).

FOULDS AND DMOCHOWSKI have immunized rabbits with filtrates of a dibenzanthracene fowl sarcoma which is not transmissible by cell-free filtrates. Filtrates have been prepared by means of graded collodion membranes in order to determine the approximate size of the antigenic particles which elicit antibodies capable of neutralizing filtrates of Rous sarcoma 1. The results indicate that the antigenic particles are larger than the infective agent of Rous sarcoma. Complement-fixation tests reveal two antigens common to the dibenzanthracene sarcoma and Rous sarcoma; one corresponds in particle size with the infective agent of Rous sarcoma, and the other is smaller. It is possible that the smaller antigenic particles result from disintegration of the larger. The relationship of the neutralizing and complement-fixing antibodies has been determined (p. 458).

DMOCHOWSKI AND KNOX have investigated the antigenic relationship of filterable and non-filterable fowl tumours using complement-fixation tests with antisera prepared in rabbits by injecting filtrates of a non-filterable sarcoma induced by 1 : 2 : 5 : 6-dibenzanthracene. The results indicate that the dibenzanthracene sarcoma and three filterable tumours (Rous sarcoma 1; Fujinami sarcoma; MH2) have a common antigen which is not species-specific. Complement-fixation tests provide an approximate measure of the infectivity of fresh filtrates of the filterable tumours. The complement-fixing antigen is therefore apparently related to the tumour-producing agent, though also demonstrable in filtrates inactivated by heat as well as in non-infective filtrates of the dibenzanthracene sarcoma (p. 466).

NAGLER describes an *in vitro* reaction between *Cl. Welchii* (Type A) lethal toxin and normal human serum. The reaction can be used as a cultural test for the identification of *Cl. Welchii*, and as a method for titrating the *Cl. Welchii* (Type A) lethal toxin and antitoxin (p. 473).

MCFARLANE, MACFARLANE, AMIES AND EAGLES have shown that dried elementary bodies of vaccinia from which 10 per cent. of lipoid has been extracted with ether or benzene can be resuspended again to the same degree of dispersity as the original virus suspension and without loss of infectivity. A further 9 per cent. of lipoid material can be extracted with alcohol-ether, and 4 per cent. of nucleic acid with sodium carbonate in both cases, with concurrent loss of infectivity. Large amounts of water are associated with each virus particle in the form of an ion atmosphere not penetrable by sucrose. The physical nature of the virus particle is discussed (p. 485).

F. V. LINGGOOD has investigated the purities of the precipitates formed when alum is added to diphtheria toxoids made from tryptic digest broths of high and low nitrogen content. It has been shown that charcoal treatment of the toxoid, prior to precipitation with alum, gives precipitates of much greater purity. Methods are suggested for preparing alum precipitates of exceptionally high purity (p. 502).

TOMPSETT has shown that the addition of hydrochloric acid to the diet increases the absorption of lead from the alimentary tract. Ammonium chloride, sodium bicarbonate and potassium iodide produce mobilization of lead on both high and low calcium intake. Recently absorbed lead may be excreted very rapidly after removal from exposure (p. 512).

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